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CHAPTER 1

1.1 Demonstrate DMC Process

Direct microbial conversion (DMC) is an approach to converting ethanol to cellulosic biomass whereby both ethanol and all required saccharolytic enzymes are produced within a single microbial system. The great potential advantage of DMC is that a dedicated process step for production of cellulase is not necessary. This feature is attractive because: 1) dedicated cellulase production per se is a significant cost, and there is some indication that this cost may have been underestimated in some studies; 2) because of the high cost of cellulase, the cost of cellulose conversion to ethanol in an SSF scenario is much greater than it would be at the levels of cellulase production characteristic of leading DMC organisms; and 3) dedicated cellulase production is an aerobic process and thus oxidizes carbon that would be converted to fermentation products in a DMC scenario, thereby decreasing the potential yield of ethanol. Because of these considerations, there can be little doubt that the ideal biological system for producing ethanol from cellulosic biomass would be a DMC system. A recent economic analysis by Lynd, Elander, and Wyman (Accepted for publication in Applied Biochem. Biotechnol.) projects that a mature process for production of ethanol from cellulosic biomass would utilize DMC, and also shows that the economic benefit of a hypothetical DMC process is greater than for any other process improvement examined.

Notwithstanding the noted potential of DMC, this cannot be considered the leading process alternative today. There are no organisms available that both produce cellulase and other saccharolytic enzymes at the required high levels and also produce ethanol at the required high concentrations and yields. Available microbial strains considered as points of departure for developing a DMC system (e.g. thermophilic bacteria) exhibit excellent production of saccharolytic enzymes, but have two key limitations: 1) it has not been established that they can produce high concentrations of ethanol and be robust under industrial conditions, and 2) available thermophilic cultures produce organic acids (principally acetic) as well as ethanol, resulting in reliably-achieved yields on the order of 70% of theoretical. This project focuses on the first of these limitations. It may be noted that other work by the P.I. addresses the second limitation.

This project seeks to demonstrate the potential feasibility of a DMC process using thermophilic bacteria. Specific issues pursuant to this goal include:

1) Can thermophiles produce and tolerate concentrations of ethanol of industrial interest (e.g. > 20 g/L)?

2) Can thermophiles be shown to behave reproducibly (which has been chronic problem in the literature), and to generally exhibit the "robustness" required of an industrial microorganism?

3) Can thermophiles be shown to simultaneously achieve high conversion of multiple carbohydrate species (cellulose, hemicellulose, soluble sugars) derived from a real-world substrate?

As presented in more detail overleaf, the project has given unambiguous positive answers to each of these questions in less than 9 months.

1.2 Ethanol Tolerance

Can thermophiles produce and tolerate concentrations of ethanol of industrial interest (e.g. > 20 g/L)?

At the outset of this project, the tolerance of the xylose-utilizing thermophile *Clostridium thermosaccharolyticum* to added (as opposed to produced) ethanol had recently been established by the P.I.'s group. Baskaran et al. (Biotechnology Progress, 1995) showed that the specific growth rate decreased approximately linearly in relation to the ethanol concentration, with 50% inhibition observed at concentrations of endogenously-added ethanol equal to 26 g/L for fermentation at 60°C and 40 g/L for fermentation at 55°C. These results indicate greater ethanol tolerance than most previous studies involving thermophilic bacteria, probably due to the selection occurring in continuous culture.

The ability of *C. thermosaccharolyticum* and perhaps other thermophiles to tolerate reasonably high concentrations of exogenously-added ethanol has not yet been fully translated, either by us or by others, into correspondingly high concentrations of endogenously-produced ethanol in an actual fermentation. While several studies have reported ethanol concentrations in excess of 20 g/L, all reports of ethanol production by thermophilic bacteria known to us are limited to concentrations < 30 g/L. Thus it would appear unlikely that the gap between the tolerance of *C. thermosaccharolyticum* to added ethanol and the maximum reported concentrations of produced ethanol are attributable to inherent biochemical limitations related to ethanol.

In light of the fact that we and many other investigators have devoted considerable effort to producing ethanol at high concentrations using thermophilic bacteria, the discussion in the previous paragraph invites the question: If ethanol is not the limiting factor in efforts to produce high ethanol concentrations with thermophiles, what is? As perhaps first observed by Monod (Ann. rev. Microbiol., 1949), growth of microbial cultures generally ceases due to either exhaustion of a required component, accumulation of an inhibitory metabolic product, or changes in the concentration of protons or other ions in the medium. In order to distinguish product inhibition from other growth-limiting factors, it is necessary to perform diagnostic experiments such as showing that growth continues when product is removed beyond that achieved in the presence of product, or that addition of product at low cell and substrate concentration is sufficient to reproduce limitation observed at high cell and

substrate concentration. For the most part, studies of ethanol production using thermophilic bacteria have not involved such diagnostic experiments. *Indeed, we know of no report of a thermophilic culture being demonstrably limited by the ethanol it produces.*

Several of these points are illustrated by recent project results. In an effort to produce ethanol concentrations commensurate with the tolerance we have documented, we undertook a series of experiments involving continuous culture of *C. thermosaccharolyticum* at progressively increasing feed xylose concentrations. At several junctures, we found that additions to or changes in the medium (presumably to supply deficient nutrients) were necessary in order to achieve complete xylose utilization. On numerous occasions, we documented robust steady-states at 75 g/L feed xylose concentration, producing ethanol at 22 to 23 g/L, acetate at 13 g/L, and virtually no lactate. Our efforts to increase the feed xylose concentration from 75 g/L to 87.5 g/L led to a decline in the culture optical density, the concentration of ethanol, and the concentration of acetate, accompanied by increases in the concentrations of unutilized xylose and lactate. A repeated experiment with elevated nutrient concentrations gave nearly identical results, suggesting that nutrient limitation was not the limiting factor. We next added ethanol to the feed of a steady-state fermentor utilizing 75 g/L xylose, resulting in a higher ethanol concentration (26 g/L) than observed in the original feed step change experiment. No adverse effect was observed, indicating that ethanol inhibition was not the limiting factor. Noting that the potassium concentration, resulting from the use of KOH to neutralize the fermentation broth, was high (generally ~19 g/L) and had risen markedly during the original step change experiment, we next added KCl to the feed of a steady-state fermentor utilizing 75 g/L xylose. The results of adding KCl were essentially the same as observed in the feed step change experiment. This provided strong evidence that potassium ion was the factor responsible for the culture's inability to reach steady-state at 87.5 g/L feed, and that ethanol inhibition played little or no role in explaining this phenomenon. The immediate significance of these results is that the tolerance of *C. thermosaccharolyticum* to produced ethanol exceeds 23 g/L even in the presence of near-inhibitory concentrations of potassium. In addition, our results underscore the point that cessation of growth and product formation should not automatically be assumed to be due to ethanol. Indeed, such cessation has been explained by factors other than ethanol (e.g. nutrient limitation, salt inhibition) in every case in which we have performed diagnostic experiments.

1.3 Reproducibility and Robustness

Can thermophiles be shown to behave reproducibly (which has been chronic problem in the literature), and to generally exhibit the "robustness" required of industrial microorganisms?

In the work with *C. thermosaccharolyticum*, reproducible results were routinely obtained. We attribute this to improved hardware, improved growth medium formulation, improved technique, and to isolation of strains that had acclimated to culture conditions. We have not yet achieved the same degree of reproducibility in studies with solid substrates (e.g. paper sludge), which are widely accepted as more challenging to work with than soluble substrates especially on a laboratory scale. However, our positive results with soluble substrates and some results on paper sludge, which showed two parallel reactors producing similar and predictable results many weeks after inoculation, provide a basis for confidence that satisfactory results can be obtained with paper sludge and other insoluble substrates.

As for robustness, our work sheds light on two factors that pose a challenge to *C. thermosaccharolyticum* : ethanol and salt. With respect to these two factors at least, this organism would appear to be quite robust. As presented by Baskaran et al (Biotech. Progress, 1995), the tolerance of *C. thermosaccharolyticum* to exogenously-added ethanol is somewhat greater than that of yeast and *Zymomonas* at temperatures used for SSF. Results from this project suggest that the salt tolerance of *C. thermosaccharolyticum* is comparable to other microorganisms, and do not give any indication of an organism that is "finicky" or hypersensitive to non-nutrient growth medium components.

Experiments using paper sludge as a substrate have shown that a coculture of *C. thermocellum* and *C. thermosaccharolyticum* is uninhibited by high concentrations of this potential commercial substrate. Batch growth has been initiated in the presence of 130 g/L sludge solids (65 g/L cellulose). It has been observed that a continuous coculture converting 17 g/L paper sludge (8.5 g/L cellulose) is able to resist upsets in pH control, feed rate and degree of reduction of the feed medium. Also, the use of stainless steel components in the reactors has not had any apparent ill effect on the thermophiles.

1.4 Simultaneous Conversion of Multiple Carbohydrates Derived from Real-world Substrates

Can thermophiles be shown to simultaneously achieve high conversion of multiple carbohydrate species (cellulose, hemicellulose, soluble sugars) derived from a real-world substrate?

This hypothesis was tested by growing a continuously fed coculture of *C. thermocellum* and *C. thermosaccharolyticum* on paper sludge (containing both cellulose and xylan) augmented with added xylose. The coculture was capable of simultaneously fermenting all of these carbohydrates in a reasonable time (20-24 hours) with endogenous production of all required saccharolytic enzymes. Conversion and closure of the carbon balance were somewhat variable between experiments. This variation was found to be largely due to inaccurate estimation of feed concentrations. Ethanol selectivity was usually on the order of 1.5 g ethanol to g organic acids, but one experiment demonstrated an ethanol selectivity of 3.6. The reason for this unusually high selectivity has not yet been determined

CHAPTER 2

Growth of *C. thermosaccharolyticum* at Elevated Substrate Concentrations

Inhibition of a Continuous Culture of *Clostridium thermosaccharolyticum* Growing at Elevated Substrate Concentrations by High Levels of Potassium Ion

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ABSTRACT

Clostridium thermosaccharolyticum was grown in continuous culture using 50 and 75 g/L xylose as carbon source. Steady states were achieved at between 19 and 40 hour residence times. Substrate utilization was in most cases complete. Ethanol production reached 22.4 g/L on the 75 g/L feed. Selectivity of ethanol with respect to organic acids was on the order of 1.8 g ethanol per g acids. Lactic acid production was minimal. A steady state at 87.5 g/L feed could not be attained. It was determined that this failure was not caused by a lack of nutrients nor by inhibition by ethanol. Rather, it was determined that above 75 g/L feed xylose the culture became inhibited by high levels of potassium ion resulting from base addition to the fermentation.

INTRODUCTION

The production of ethanol from cellulosic substrates is considered to be a viable option for the development of a renewable source of transportation fuel. Ethanol's utility is similar to that of gasoline, and it is conceivable that it could be produced on a scale commensurate with gasoline usage in North America[1]. The development of a cellulosic biomass fuel ethanol industry in North America is expected to provide additional advantages in the areas of: employment; agriculture; urban air quality; reduced net release of CO_2; balance of trade and energy security [1,2].

Direct microbial conversion (DMC) is an approach to converting cellulosic biomass to ethanol whereby both ethanol and all required saccharolytic enzymes are produced within a single microbial system. The great potential advantage of DMC is that dedicated process steps for production of cellulase and fermentation of pentose sugars are not necessary. Available microbial strains considered as points of departure for developing a DMC system (e.g. thermophilic bacteria) exhibit excellent production of saccharolytic enzymes and excellent utilization of a variety of substrates. However, DMC cannot be considered the leading process alternative today because there are no organisms available that both produce cellulase and other saccharolytic enzymes at the required high levels and also produce ethanol at the required high concentrations and yields. The thermophiles have two key limitations: 1) it has not been established that they can produce high concentrations of ethanol and be robust under industrial conditions, and 2) available thermophilic cultures produce organic acids (principally acetic) as well as ethanol, resulting in reliably-achieved yields on the order of 70% of theoretical. This work addresses the first of these concerns.

The tolerance of the xylose-utilizing thermophile *Clostridium thermosaccharolyticum* to added (as opposed to produced) ethanol has recently been reported by Baskaran et al. [3]. In this work it was shown that the specific growth rate decreased approximately linearly in relation to the ethanol concentration, with 50% inhibition observed at concentrations of endogenously-added ethanol equal to 26 g/L for fermentation at 60°C and 40 g/L for fermentation at 55°C. These results indicate greater ethanol tolerance than most previous studies involving thermophilic bacteria, probably due to the selection occurring in continuous culture.

The ability of *C. thermosaccharolyticum* and perhaps other thermophiles to tolerate reasonably high concentrations of exogenously-added ethanol has not yet been fully translated, either by us or by others, into correspondingly high concentrations of endogenously-produced ethanol in an actual fermentation. While several studies have reported ethanol concentrations in excess of 20 g/L, all reports of ethanol production by thermophilic bacteria known to us are limited to concentrations of less than 30 g/L. Studies of a decade ago or more ago suggested that microorganisms might exhibit greater sensitivity to ethanol that is endogenously-produced rather than exogenously-added due to intracellular accumulation of ethanol. However, this contention has been refuted on both experimental and theoretical grounds in a series of more recent papers that has gone unchallenged. Thus it would appear unlikely that the gap between the tolerance of *C. thermosaccharolyticum* to added ethanol and the maximum reported concentrations of produced ethanol are attributable to inherent biochemical limitations related to ethanol.

The discussion in the previous paragraph invites the question: If ethanol is not the limiting factor in efforts to produce high ethanol concentrations with thermophiles, what is? As perhaps first observed by Monod [4], growth of microbial cultures generally ceases due to either exhaustion of a required component, accumulation of an inhibitory metabolic product, or changes in the concentration of protons or other ions in the medium. In order to distinguish product inhibition from other growth-limiting factors, it is necessary to perform diagnostic experiments such as showing that growth continues when product is removed beyond that achieved in the presence of product, or that addition of product at low cell and substrate concentration is sufficient to reproduce limitation observed at high cell and substrate concentration. For the most part, studies of ethanol production using thermophilic bacteria have not involved such diagnostic

experiments. Indeed, we know of no report of a thermophilic culture being demonstrably limited by the ethanol it produces.

MATERIALS AND METHODS

Bacterial Strains

Clostridium thermosaccharolyticum DHG8, a descendant of strain HG8 was used for these experiments. *Clostridium thermosaccharolyticum* strain HG8, a low acid producing strain developed at MIT through nonspecific mutagenesis, was originally provided by Prof. A.L. Demain of M.I .T. Since delivery, strain HG8 has been cultivated for more than a decade at Dartmouth College.

For culture maintenance, single colony isolates are identified after preparing a dilution series of anaerobic agar roll tubes (Bellco 18 x 150 mm) maintained under 5 psi N_2 atmosphere with aluminum crimped-sealed rubber septa. Agar at 45 g/l is added to the carbohydrate solution of the growth medium. Concentrated nutrient solutions are added once the carbohydrate solution has cooled to 60-70°C. After mixing, the diluted broth sample is added, the tube mixed by inversion, and thereafter spun using a modified Bellco (Vineland NJ) tube spinner for 5 minutes or until cool. Single isolates are lifted from the solid medium using tuberculin syringes. The picked colony is transferred to liquid medium and incubated 24 hrs. or until OD reaches 0.8. The suspension obtained is transferred as 2 mL aliquots into 5 mL serum bottles (Wheaton Glass, Vineland, NJ) containing 2 mL sterile glycerol under 5 psi N_2. The glycerol-protected cultures are then stored at -80 °C.

Medium Formulation

All media is made using singly distilled water. All chemicals are of reagent grade or better and are usually obtained from Sigma Chemical Co. (St. Louis, MO) or J.T. Baker (Jackson, TN). The carbohydrate used was xylose (Sigma Chemical Co., St. Louis, MO) in concentrations of between 50 and 87.5 g/L. To make the medium, up to six separate solutions are prepared. Solution A contains the carbohydrate, the anti foam Mazu at 10 µl per g of xylose, rezazurin at 5 mL/L and yeast extract (0 to 5 g/L) in distilled water corresponding to 90% of the desired final volume. When large volumes are prepared, the xylose is autoclaved separately as a concentrated solution. Solution B is prepared as a 25-fold concentrate to give final concentrations of 2 g/L citric acid tripotassium salt, 1.25 g/L citric acid free acid monohydrate, 1 g/L sodium sulfate, 3 g/L potassium phosphate

monobasic, 2.5 g/L sodium bicarbonate. Solution C is prepared as a 50-fold concentrate to give a final concentration of 3.5 g/L ammonium chloride. Solution D is also a 50-fold concentrate to give final concentrations 1 g/L magnesium chloride hexahydrate, 0.5 g/L calcium chloride dihydrate, 0.1 g/L ferric chloride tetrahydrate, 1 g/L L-cysteine hydrochloride monohydrate. Solution E is a 50-fold concentrate of vitamins to give 5 mg/L pyridoxamine dihydrochloride, 1 mg/L p-amino benzoic acid, 0.5 mg/L d-biotin, 0.5 mg/L vitamin B_{12} and 1 mg/L thiamin. The vitamin solution is stored at 4°C in the absence of light for up to six months.

The five or six medium solutions are sterilized separately. Most solutions are autoclaved at 121 °C for a minimum of 20 minutes. Large volumes (18 L) of A solution are autoclaved for 90 minutes. Solution E is sterilized by filtration through a 0.2 um filter (Gelman Sciences, Ann Arbor MI). In batch experiments, tube-to-tube variability is reduced by combining solutions C through E with solution B and adding a single 10-fold concentrate to solution A.

Measurement of Cell Mass

Cell mass is determined optically, or by dry weight. Optical density of a cell suspension grown on soluble substrates is measured in a 13 mm diameter Pyrex tube at 660 nm using a Spectronic 21D (Milton Roy, Riviera Beach, FL) and a water blank. Absorbance measurements are only considered accurate over the range 0.010 - 0.800 absorbance units. Cell suspensions measuring above 0.8 units are sufficiently diluted using distilled water.

Dry weight measurement utilizes 0.2 or 0.45 um polycarbonate Nucleopore (Gelman, Ann Arbor, MI) filters of 47 mm diameter. The filters are stored in a 72°C desiccated oven, weighed prior to use, and then a 1-25 mL sample of cell-containing broth is passed through them using a vacuum filtration unit. The filter cake is washed 1-2 times with distilled water and then dried to constant weight in the 72°C desiccated oven.

Measurement of Alcohols, Organic Acids, and Carbohydrates

Concentrations of alcohols, organic acids, and carbohydrates are determined via HPLC using a Bio-Rad HPX-87H column (Bio-Rad, Richmond, CA) at 55-60°C with a 0.01 N sulfuric acid running eluent circulated at 0.5 mL/min. by a pulse-damped mini Pump (Milton Roy, Riviera Beach, FL). Acidified samples of 10 µL are injected in line using a VICI EQ-60 six port stainless/viton valve (Valco Instruments, Houston, TX) using an

Alcott 728 auto injector (Alcott Chromatography, Norcross, GA) with one injection and zero rinses per vial. Concentrations were based on measurements of refractive index, detected by an Altex T56 (Beckman Instruments, Palo Alto, CA) differential refractometer using an eluent reference. Signals from the refractometer are collected every 2.5 sec through an 8 bit DA board installed in an IBM PS 2/30 running LabTech Notebook and LabTech Chrom software. Concentrations are determined by peak height rather than area.

Samples are prepared for HPLC analysis by acidifying 900 µL samples with 100 µL of 10 % wt/vol. H_2SO_4 followed by centrifugation at 15,900 x g for 10 minutes. The clarified supernatant is transferred in 700 µL aliquots to glass or polypropylene injection vials (Alcott Chromatography, Norcross, GA), capped, and analyzed or stored refrigerated until the time of analysis. Reference concentrations are provided by a standard solution made by combining a 900 mL solution containing 1.000 g/l D(+)cellobiose, 1.000 g/l citric acid monohydrate, 1.000 g/l b-D(+)glucose, 1.000 g/l, D(+)xylose, 1.095 g/l L(+)lactic acid lithium salt, 1.828 g/l formic acid potassium salt, 6.830 g/l sodium acetate, 1.000 g/l MOPS sodium salt, 5.00 g/l ethanol (6.33 mL absolute) with a 100 mL solution containing 10 % wt/vol. sulfuric acid. The acidified standards are stored at 4°C for up to 1 year. One standard solution is injected for about every six samples analyzed.
When concentrations of carbohydrate or ethanol exceed 10 g/l or 20 g/l respectively, the samples are sufficiently diluted using 1 % wt/vol. sulfuric acid.

Batch Cultivation Systems

Batch cultivations were performed both with and without active pH control.
Batch fermentations without pH control are performed in 100-250 mL serum bottles (Wheaton Glass, Vineland, NJ) fitted with an 13 mm ID anaerobic rubber septum (Bellco Glass, Vineland, NJ) and secured by an aluminum crimp seal. The serum bottles are prepared by filling with the appropriate A solution of MTC medium, capping, and evacuating and flushing with 5 psi N_2 four times prior to autoclaving. Following autoclaving, the additional medium components and the inoculum are added to the serum bottle using syringes. Incubation is done without agitation in a 55 °C gravity convection incubator (VWR Scientific, Boston, MA). During the course of fermentation, samples are removed asceptically from the serum bottles using 1-20 cc syringes fitted with 18 - 23 g needles.

Continuous Cultivation Systems

Continuous fermentation of soluble substrates are performed in custom 250 mL working volume glass reactors (#03179102, NDS Technologies, Vineland, NJ) with three integral baffles. The jacketed reactors are fitted with a shaped neoprene cap which has four 5-mm passages fitted with Pyrex heavy wall glass tubing for feed addition, caustic addition, fermentor sampling and overflow. A fifth 5-mm port holds a 6 mm x 150 mm pH probe (Cole-Parmer, Niles, IL), and two 18-mm passages are fitted for inoculation and temperature measurement. The cap and all the fittings are friction fit. Mixing is provided by a Teflon stir bar powered by a magnetic stir plate (Cole-Parmer, Niles, IL). All materials in contact with the fermentation broth are composed of glass, Teflon, or neoprene. Nylon connectors are used in caustic lines. Nickel plated brass Luer connectors (Engineers Express, Medway, MA) were used in feed, sampling, and effluent lines and were flame sterilized when changing feed and effluent reservoirs.

Medium is pumped into the fermentor using a variable speed digital peristaltic pump (Cole-Parmer, Niles, IL) with 0.8 mm ID neoprene tubing. Samples of the medium are drawn asceptically from flow through cells (#06069102, NDS Technologies, Vineland, NJ) positioned upstream of the medium drip tube. A fixed speed 2 rpm peristaltic pump (Cole-Parmer, Niles, IL) is used to meter concentrated KOH or Ca(OH)2 slurry through neoprene lines. PID control of fermentor pH is performed by an Applicon ADI 1020 (Applicon Dependable Instruments, Foster City, CA). Under normal circumstances, pH control is unidirectional and the pH setpoint, is maintained to ± 0.5 pH units. Reactor level is maintained at 330 ± 15 mL by gas pressure generated in the course of fermentation with the two phase effluent mixture being driven into an aseptic, vented 4 liter Erlenmeyer flask.

The reactor is typically autoclaved with the sampling drip tubes attached, although a luer connector between drip tubes and reactor permits a change of drip tubes in the event of a clog.

Prior to filling the reactor with fresh medium, the majority of the reactor contents are blown out of the reactor using N_2 by applying gas through the effluent reservoir and discharging liquid through the sample line. After emptying, the gas source is moved to the sampling drip tube, the effluent line is opened, and N_2 is bubbled through the reactor for 10-20 min. to establish anaerobisis. Fresh medium is introduced through the medium inlet line until liquid begins to discharge through the overflow line and into the effluent reservoir. At this point, the sample line is clamped shut to stop gas flow. With the pH adjusted and anaerobisis established, the appropriate inoculum is introduced via the

flame-sterilized inoculation septum. The overflow line is left open as a vent for the gases of fermentation.

RESULTS

Several experiments were carried out at a feed concentration of 50 g/L xylose. Steady states were repeatably achieved at different residence times. Table I summarizes results from these experiments. Characteristics of the growth medium that had been changed from previous work to improve growth at high substrate concentrations included the use of citric acid chelating agents, the use of bicarbonate as a buffer, reduction of sulfate ion concentration and the addition of 5 g/L yeast extract. Similar results for steady state experiments at 75 g/L are reported in table II. It was found that steady state growth at 75 g/L correllated with the use of medium which was relatively fresh and had not been allowed to sit at room temperature for an excessive amount of time.

Steady state at a feed concentration higher than 75 g/L was not achieved. Several attempts were made but results were invariably negative. Figure 1 documents the failure of a continuously operating reactor caused by a step change in feed concentration from 75 g/L to 87.5 g/L. As shown in the figure, shortly after the step change in input feed concentration, it was observed that there was a decline in the culture optical density, the concentration of ethanol, and the concentration of acetate, accompanied by increases in the concentrations of unutilized xylose and lactate.

Experiments were conducted to determine the cause of the inability to convert a higher feed substrate concentration. First, it was hypothesized that nutrient limitation may have been responsible. This was tested by doubling the yeast extract to 10 g/L and increasing the levels of all other nutrients proportionately to the increase in feed xylose concentration (16.7%). Figure 2 shows the steady-state utilization of 75 g/L xylose feed before time zero. Xylose utilization was essentially complete and was accompanied by production of 20.2 g/L ethanol. At time zero, the feed xylose and nutrient concentrations were increased leading to rapid wash-out of the culture. The pattern observed is similar to that reported for the normal nutrient levels experiment.

Second, it was hypothesized that ethanol inhibition was responsible for the wash-out at 87.5 g/L xylose. As shown in Figure 3, the feed to a steady-state fermentor utilizing 75 g/L xylose was altered by the step addition of 4.2 g/L ethanol to the feed stream at time

zero. The level of ethanol added to the feed represents the additional ethanol production which would be expected from complete utilization of the previously attempted increase in feed xylose (12.5 g/L). The measured ethanol concentration in the fermentor is the total of produced and added ethanol. Xylose utilization remained high at all times, with a transient peak of ~1 g/L. At steady-state, the ethanol concentration was 26.3 g/L, with concentrations of all other nutrients unaltered relative to their pre-step levels. The negligible impact of adding ethanol is compelling evidence that ethanol inhibition (at present production levels) is not a major constraint to higher xylose utilization.

Finally, the potential for inhibition due to the neutralization agent, potassium hydroxide, was investigated. Steady-state potassium levels were calculated based on volumetric consumption rates of 15% KOH at 50 g/L and 75 g/L feed xylose levels. These numbers are shown in figure 4. Note how the increase in base demand appears to be non linear with increase in feed concentration. The concentration of potassium in the fermentor at various time points during a failed attempt at 87.5 g/L xylose feed was estimated using a finite difference approximation and the material balance equation for potassium. These estimated concentrations are included in figure 1. The peak concentration of potassium in the fermentor with 87.5 g/L feed xylose was estimated to be about 26.4 g/L, which is higher than the steady-state level for 75 g/L xylose by 7.3 g/L.

Shown in Figure 5, the feed to a steady-state fermentor utilizing 75 g/L xylose was altered by the addition of 7.3 g/L potassium (as the chloride) at time zero. The addition of potassium resulted in rapid wash-out that closely mirrored the response of the fermentor to the step increase in feed xylose concentration from 75 g/L to 87.5 g/L.

DISCUSSION

In this work with *C. thermosaccharolyticum*, reproducible results were routinely obtained. Tables I and II show that reliable steady states at 50 and 75 g/L feed concentrations were repeatably achieved and maintained for durations of many residence times. Lack of reproducibility has been a recurring problem in reports of thermophile growth.

The data in Figures 1,2,3 and 5 provide strong evidence that potassium ion was the factor responsible for the culture's inability to reach steady-state at 87.5 g/L feed, and that ethanol inhibition played little or no role in explaining this phenomenon. The immediate

significance of these results is that the tolerance of *C. thermosaccharolyticum* to produced ethanol exceeds 23 g/L even in the presence of near-inhibitory concentrations of potassium. In addition, these results underscore the point that cessation of growth and product formation should not automatically be assumed to be due to ethanol. Indeed, such cessation has been explained by factors other than ethanol (e.g. nutrient limitation, salt inhibition) in every case in which we have performed diagnostic experiments.

Figure 4 shows that the increase in base demand is not linear with increasing substrate concentration. This may be due to exhaustion of the buffering capacity of the medium. This non linearity could potentially pose a serious obstacle to achieving higher substrate concentrations. It is possible that in the longer term, genetic engineering to eliminate organic acid production by ethanol producing thermophiles may obviate the problem of inhibition caused by base addition.

This study also demonstrates that *C. thermosaccharolyticum* is in many respects a robust organism. This work sheds light on two factors that pose a challenge to *C. thermosaccharolyticum* ethanol and salt. With respect to these two factors at least, this organism would appear to be quite robust. As presented by Baskaran et al [3], the tolerance of *C. thermosaccharolyticum* to exogenously-added ethanol is somewhat greater than that of yeast and *Zymomonas* at temperatures used for SSF. Table III presents the data we have gathered thus far on salt inhibition for *C. thermosaccharolyticum.* These data suggest a salt tolerance comparable to other microorganisms, and do not give any indication of an organism that is "finicky" or hypersensitive to non-nutrient growth medium components.

CONCLUSION

The results of this study provide confirmation that ethanol inhibition is not a constraining factor for higher utilization of xylose at concentrations greater than 75 g/L and production of ethanol at concentrations greater than 23 g/L. Potassium inhibition satisfactorily explains the apparent inability of *C. thermosaccharolyticum* to ferment higher feed xylose concentrations. These results suggest that the endogenous ethanol tolerance may be consistent with estimates for exogenous ethanol tolerance [3]. To the best of our knowledge, potassium inhibition at high feed xylose concentrations has not been documented previously for *C. thermosaccharolyticum.* It seems likely that salt inhibition may explain failed attempts by various investigators to increase substrate utilization by thermophiles, which were often attributed to ethanol inhibition. Information gathered thus far from the literature suggests that the potassium tolerance of *C. thermosaccharolyticum*

is rather high relative to other microbial systems. This is consistent with *C. thermosaccharolyticum* being "robust," a key requirement for an industrial microorganism.

ACKNOWLEDGMENTS

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FIGURES AND TABLES

Table I

Continuous Fermentation of 50 g/L Xylose with *C. thermosaccharolyticum* DHG8
(all concentrations in g/L)

FERMENTOR	**R 17**	**R17**	**R18**	**R20**	**R21**
Residual xylose	0	0	0.4	0.5	0.5
Lactate produced	0	0	1.4	0	0
Acetate produced	7.9	7.9	8.4	7.4	8.81
Ethanol produced	13.6	14.3	15.4	14.2	16.42
Absorbance (660 nm)	1.6	4.2	4.3	4.0	3.9
Nominal residence time (h)	40	20	24	24	19
Dilution rate due to feed (h^{-1})	0.0237	0.0494	0.0424	0.0406	0.0529
Consolidated dilution rate (h^{-1})	0.0256	0.0525	0.0484	0.0404	na
Base flow rate (mL/h)	0.66	1.04	2.06	1.15	1.08
Corrected feed xylose	46.23	47.1	43.8	45.8	47.2

Table II

Continuous Fermentation of 75 g/L Xylose with *C. thermosaccharolyticum* DHG8 (all concentrations in g/L)

Residual xylose	0.6
Lactate produced	0
Acetate produced	11.4
Ethanol produced	22.4
Absorbance (660 nm)	5.6
Nominal residence time (h)	19
Dilution rate due to feed (h^{-1})	0.0529
Consolidated dilution rate (h^{-1})	0.0581
Base flow rate (mL/h)	2.22
Corrected feed xylose	66.56

Table III

Tolerance to Monovalent Cations

Organism	Cultivation Modes	Cation	Tolerance (g/L)	Reference
Digester Sludge	batch	Na^+	13.8[a]	[5]
M. thermophila	batch	Na^+	12.6 to 14.9[a]	[6]
Z. mobilis	batch	K^+	10.0[a]	[7]
M. thermophila	batch	Na^+	13.8[b]	[8]
			27.6[a,c]	
C. thermosacch.	continuous	K^+	19.0[d]	Baskaran this work

a: 100% inhibition
b: M = 0.5 Mmax
c: Pregrown on marine media
d: M= 0.05 Mmax
e: Maximum cation concentration at which metabolism observed

Figure 1

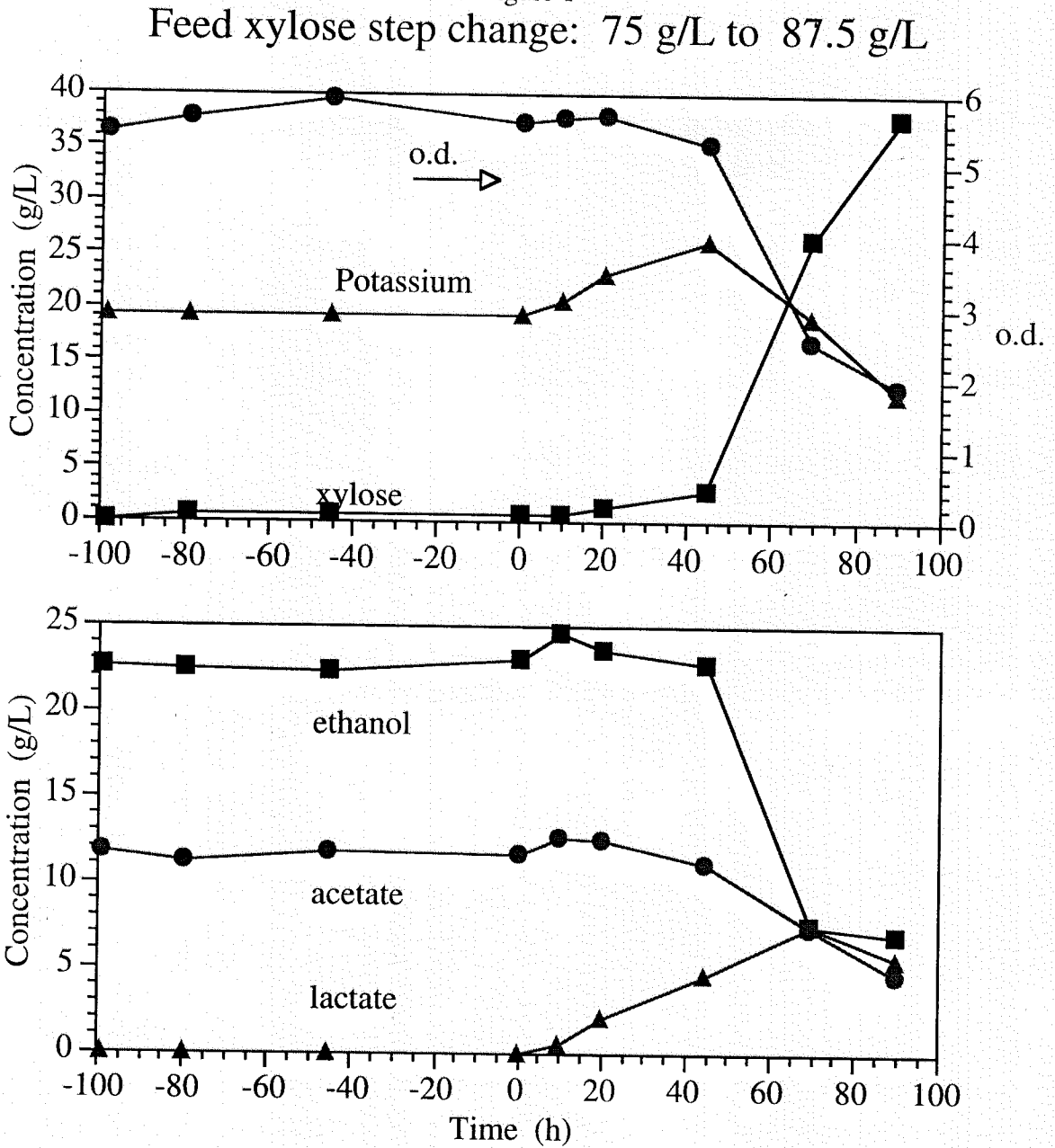
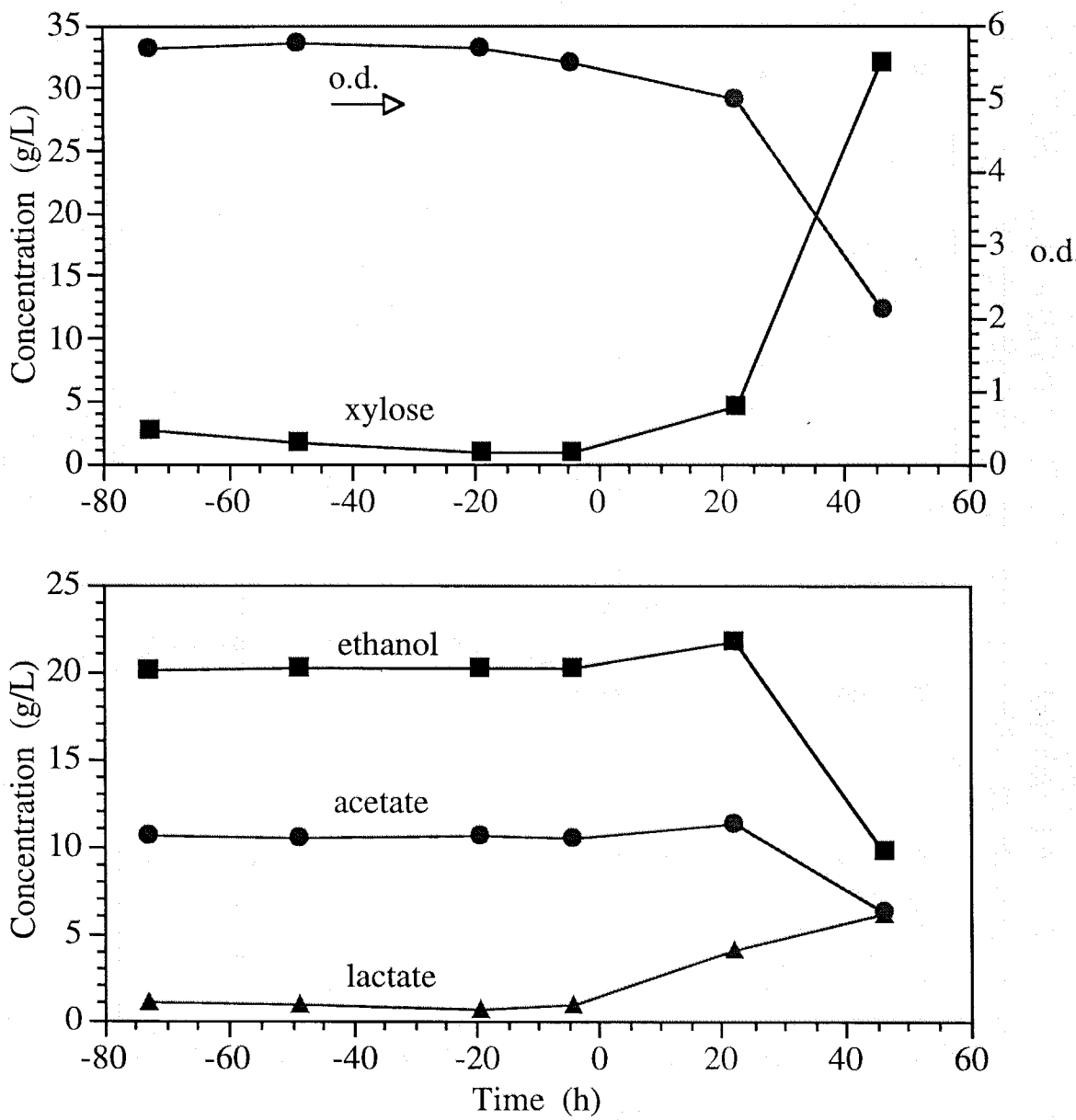

Feed xylose step change: 75 g/L to 87.5 g/L
Concentration (g/L)
o.d.
Potassium
xylose
ethanol
acetate
lactate
Time (h)

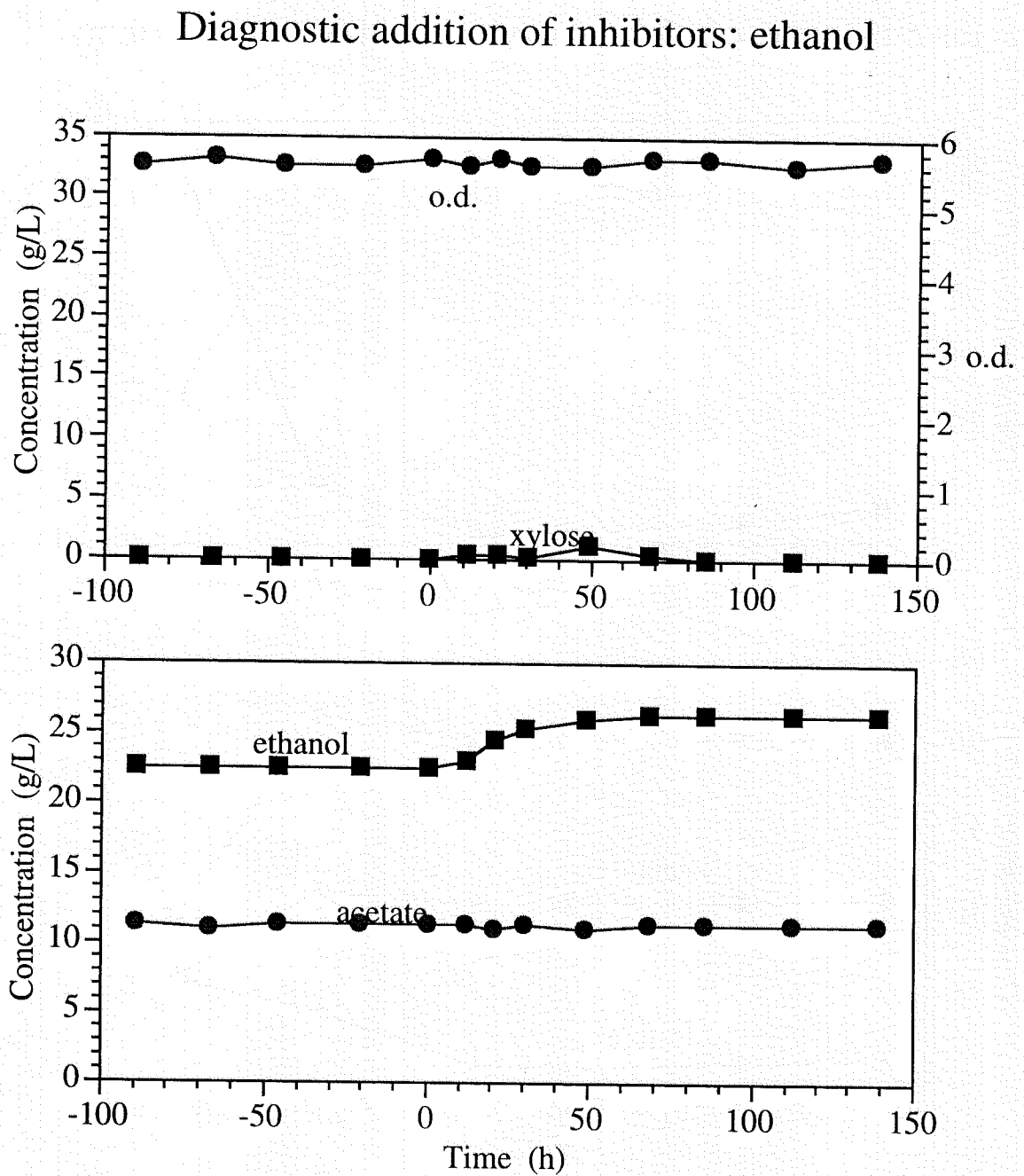
Figure 2

Fermentation of 87.5 g/L xylose with scaled nutrients



Feed xylose increased from 75 g/L to 87.5 g/L at time = 0 h.
Dilution rate - 0.0406 h^{-1}

Figure 3



Feed ethanol concentration increased from 0 to 4.2 g/L at time = 0 h.
Feed xylose concentration unchangedat 75 g/L.
Latate production not detected.

Dilution rate = 0.0581 h^{-1}

Figure 4

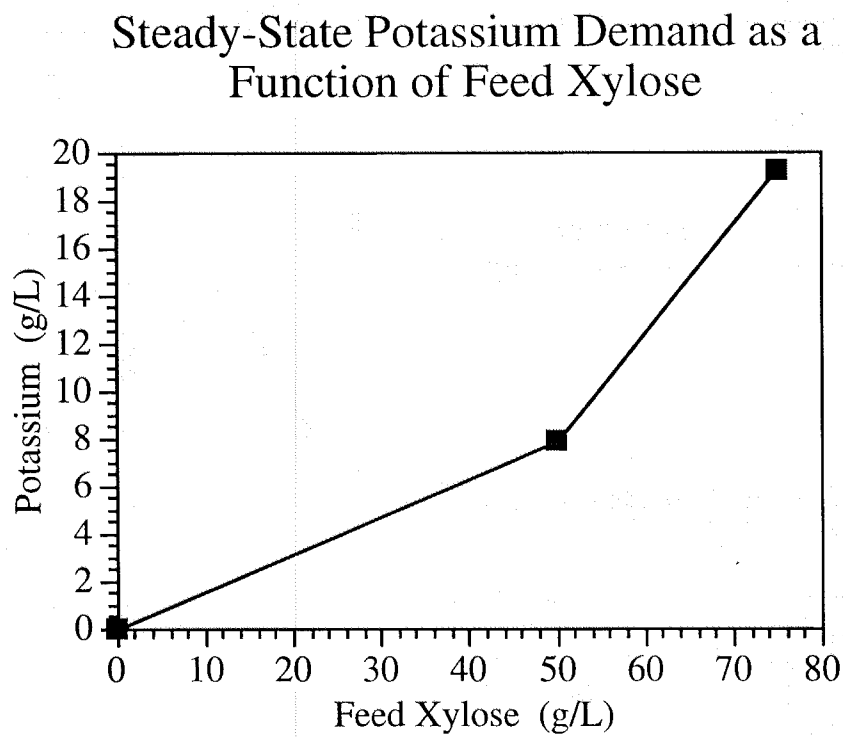
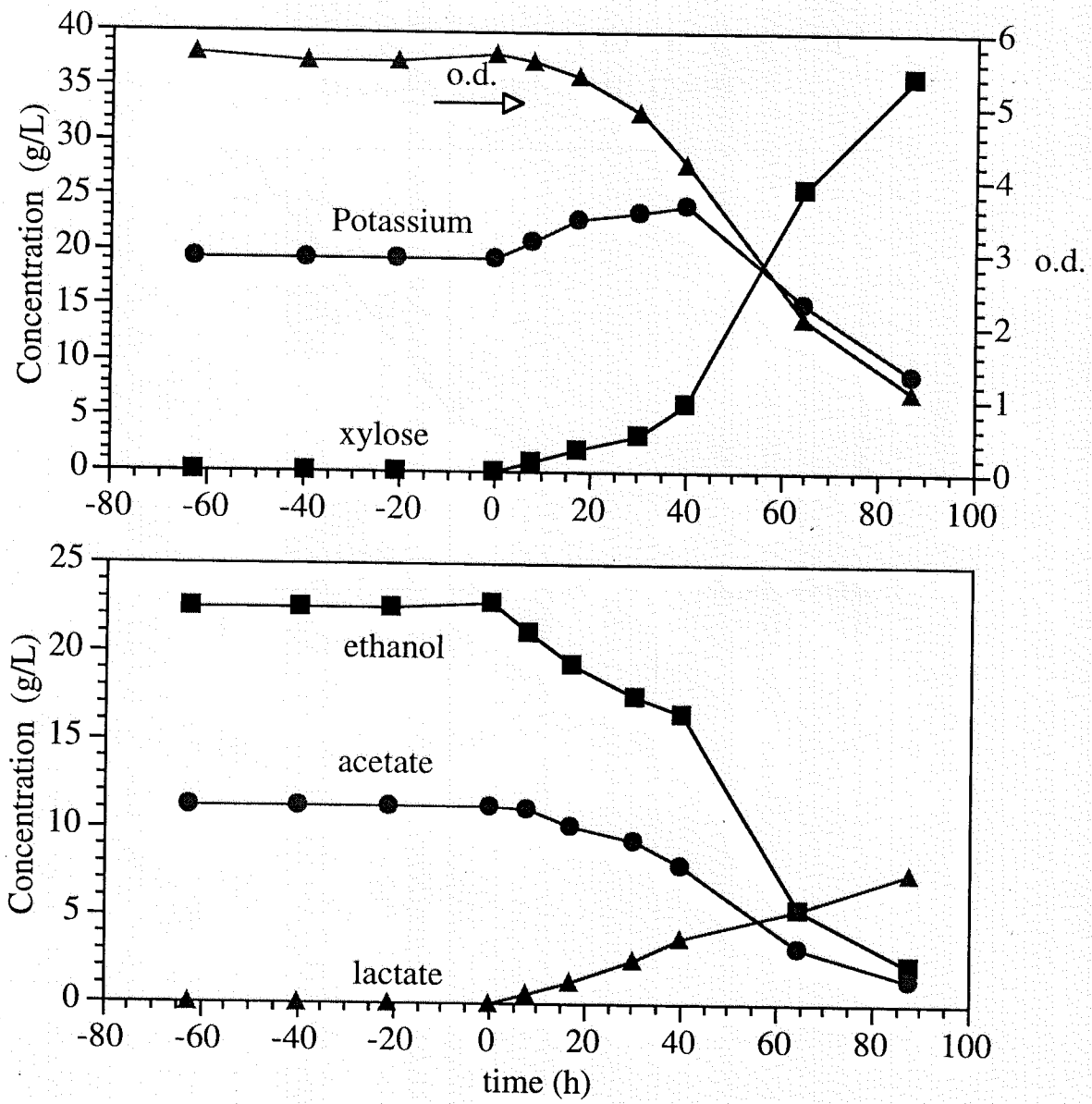

Steady-State Potassium Demand as a Function of Feed Xylose
Potassium (g/L)
0
2
4
6
8
10
12
14
16
18
20
0
10
20
30
40
50
60
70
80
Feed Xylose (g/L)

Figure 5

Diagnostic addition of inhibitors: potassium



7.3 g/L potassium (as KCl) added to feed at time = 0
Feed xylose concentration unchanged at 75 g/L

Dilution rate = 0.0581^{h-1}

2.2 Use of Calcium Hydroxide as a Neutralizing Agent for Continuous Culture

Having identified potassium ion as being responsible for inhibition of the culture, experiments were undertaken to circumvent this inhibition using different bases. Two approaches were tried. First, increasing the buffering capacity of the medium was tried as a means of reducing base demand. An experiment was conducted using MOPS as a representative buffer. Although increasing the buffer capacity does reduce base demand, it was determined that the return in base demand savings was relatively minor compared to copious amount of additional buffer required.

Next, several bases were considered as replacements for KOH. Previous experience with NaOH in continuous fermentation of 50 g/L and 75 g/L xylose indicated that this was a poor choice. Ammonia was ruled out since ammonia inhibition has been observed at concentrations of just a few g/L (D. Hogsett, unpublished; L. Lynd, doctoral thesis). We thus focused our efforts on $Ca(OH)_2$ and $Mg(OH)_2$. Several lines of thought suggested that these common industrial bases might be less inhibitory : 1) their associated cations are ubiquitous in cells, and thus might be expected to have less of an effect than more exotic neutralizing agents (e.g. LiOH, RbOH); 2) their dibasic character leads to a lower concentration of cations per amount of neutralization delivered; and 3) the limited solubility of carbonate salts of Ca^{++} and Mg^{++} suggests that formation of $CaCO_3$ and $MgCO_3$ precipitates might further limit the concentration of potentially inhibitory dissolved cations. Chemical tests were carried out comparing Mg(OH)2, CA(OH)2 and KOH as neutralizing agents for a system continuously fed with acetic acid. The results indicated that while all three bases were of roughly equal effectiveness as neutralizing agents, that $Ca(OH)_2$ was the most effective.

Use of $Ca(OH)_2$ required careful design of the base feeding system, paying special attention to the diameter of tubing and fittings. It was found that with properly sized pumps and tubing that a slurry with high solids concentration could be fed to the reactors with only minor flow inconsistencies. Also, the increased solids content of the fermentors caused difficulties with conventional stir bar agitation. It was found that stir bars enclosed in a circular ring made of Teflon worked the best of the magnetic designs. Direct drive is the preferred solution, however.

There was concern that saturating the reactor with poorly soluble cations might make needed nutrients unavailable or otherwise inhibit the culture. This concern was addressed

directly by conducting continuous fermentations using the base slurries. In general it was found that the use of Ca(OH)2 posed no serious problems to the culture. Results from experiments carried out at 10 and 50 g/L xylose feed are presented in table I below. Steady state growth at 75 g/L feed xylose has not yet been achieved but it appears possible from results so far.

A readily-evident precipitate was visible in the samples from the calcium neutralized reactors which was not present in their potassium counterparts. This precipitate bubbles when added to acid, suggesting that it may be calcium carbonate. Regardless of the anionic portion of this compound, it is strongly suspected that this is a calcium salt, and that it is effectively reducing both the ionic strength and the number of dissolved species in solution in the calcium fermentor. This precipitate has also made it difficult to measure the optical density of the fermentor, as the high volume of suspended solids makes the concentration of bacteria relatively minor by comparison

Table I: Output of $Ca(OH)_2$ buffered fermentor

	10 g/L Xylose Average	Std. Dev.	**50 g/L Xylose** Average	Std. Dev.
% sub. conv.	94.8%	7.9%	99.3%	0.1%
% C recovery	107.3%	12.1%	89.5%	9.5%
EtOH Yield	32.3%	3.2%	28.2%	3.0%
Acetate	1.90 g/L	0.28 g/L	7.27 g/L	0.74 g/L
Lactate	0.71 g/L	0.53 g/L	0.58 g/L	0.23 g/L
Ethanol	3.06 g/L	0.41 g/L	13.98 g/L	1.48 g/L

CHAPTER 3

DMC of a Potential Industrial Substrate

Thermophilic Conversion of Paper Sludge to Ethanol Using a Coculture of *Clostridium thermocellum* and *Clostridium thermosaccharolyticum*

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ABSTRACT

Primary clarifier sludge from a paper mill producing high quality white paper from kraft pulp was converted to ethanol using Direct Microbial Conversion (DMC) in batch, fed batch and continuous operation. The thermophilic bacteria *Clostridium thermocellum.* and *Clostridium thermosaccharolyticum* were grown at substrate concentrations between 5 and 65 g cellulose/L. In batch growth, low substrate concentrations (5-10g/L) resulted in 85% conversion in 50 to 100 hours and ethanol concentrations of up to 3 g/L. At higher substrate concentrations, ethanol concentrations in fed batch experiments could reach 10g/L after several days. Conversion in fed batch at higher substrate concentrations was on the order of 50-60%. In continuous operation, 17 to 20 g/L paper sludge supplemented with 5 g/L xylose was converted to ethanol and other products at residence times of 20 to 24 hours. Conversion ranged from 77 to 94 %. Selectivity of ethanol to organic acids ranged from 1.4 to 3.6 on a mass basis.

INTRODUCTION

The production of ethanol from cellulosic substrates is considered to be a viable option for the development of a renewable source of transportation fuel. Ethanol's utility is similar to that of gasoline, and it is conceivable that it could be produced on a scale commensurate with gasoline usage in North America[1]. The development of a cellulosic biomass fuel ethanol industry in North America is expected to provide additional advantages in the areas of: employment; agriculture; urban air quality; reduced net release of CO_2; balance of trade and energy security [1,2].

Although the development of a cellulosic biomass ethanol industry holds promise, there are several obstacles yet to be overcome. One obstacle is the high cost of cellulase used to hydrolyze the cellulose to fermentable sugars. The cost of cellulase impacts the cost of the entire bioconversion process[1,3]. Secondly, pretreatment technologies are expensive and poorly understood[4-7]. To be hydrolyzed and subsequently fermented to ethanol, most cellulosic substrates must first be pretreated. Current pretreatment technologies suffer from problems of corrosion, high energy requirements, production of microbial growth inhibitors and environmental liabilities. Thirdly, much of the emerging technology addressing these and other problems is unproved on a commercial scale. A proving ground is needed for new technologies that can provide an entry point for scale-up and commercialization.

Thermophiles:

The obstacle of cellulase production can be addressed by making use of thermophilic bacteria that both produce their own cellulases and ferment the hydrolyzed cellulose to ethanol[1,3,8]. Thermophiles thus eliminate the problem of high enzyme costs. Much work has been published on the growth and characteristics of thermophiles [e.g. 9-15], but few have investigated growing these organisms on realistic, industrial substrates[14-17]. Previous work at the Thayer School of Engineering has for the first time demonstrated a coculture of *C. thermocellum* and *C. thermosaccharolyticum* to be stable in continuous culture on insoluble cellulose[14]. This coculture system is of great interest not only because it produces its own cellulase but also because it also can consume both pentose and hexose type sugars. This increases the potential yield of products above that of a more conventional simultaneous saccharification and fermentation (SSF) system using yeast.

Paper Sludges:

A second opportunity that is studied in this project is the use of waste cellulose in the form of primary clarifier sludge from a paper mill. The use of paper sludge has the potential to address the two obstacles of pretreatment and prohibitive commercial investment requirements. Paper sludge is an inexpensive source of highly processed substrate uniquely suitable for conversion to ethanol. The composition of paper sludge varies from one paper mill to the next, but in general they all contain cellulose, hemicellulose and an inert fraction made up of clays and minerals that are used as fillers and coatings for the paper. The sludge from paper mills producing white paper contains low levels of lignin. Because of this, the amount of cellulase required to process paper sludge is less than that required to process other ligno-cellulosic feeds. Preliminary work using SSF at the Thayer School and elsewhere [18- 21] have shown some paper sludges to be highly reactive while requiring little or no pretreatment prior to hydrolysis. Thus, making use of this substrate eliminates the several difficulties associated with pretreatment. Paper sludge also offers unique benefits when looking ahead to pilot plant operation or commercialization. Substantial process simplifications can be realized using paper sludge because there is little or no need for pretreatment. Steam supply and waste water treatment infrastructures are typically available at the mills where sludges are produced. Combining a paper sludge feed stock with a DMC fermentation process has the potential to greatly simplify the overall conversion process (see figure 1). As an additional incentive to using paper sludge, the current practice of dumping paper sludge in land fills is an expense for paper producers. In locations with high land fill costs, the

reduction of this waste volume via conversion to ethanol would result in substantial savings.

The combined impacts of low or negative substrate cost, high reactivity, simplified processing and supply volumes compatible with a small scale commercial operation make a compelling case that paper sludge can provide the needed proving ground for the production of ethanol from cellulosic biomass using DMC.

Continuous processing is particularly advantageous for the conversion of paper sludge. Paper sludge slurries are viscous and the fibers clump and dewater readily, making it difficult to agitate the slurry at high concentrations. However, recent studies at the Thayer School and elsewhere[18-21] have demonstrated that paper sludge readily liquefies upon hydrolysis. Thus continuous processing--steadily adding new sludge to a reactor filled with partially converted substrate--will greatly increase the maximum substrate concentration attainable.
This work investigates thermophilic conversion of paper sludge to ethanol in batch and continuous processes.

MATERIALS AND METHODS

Organisms:

Clostridium thermocellum TEX-5, a descendant of ATCC 27405 was used in coculture with *Clostridium thermosaccharolyticum* DHG-8, a descendant from strain HG-8. HG-8 is a low acid producing strain developed at MIT through nonspecific mutagenesis. *Clostridium thermocellum* strain ATCC 27405 and *Clostridium thermosaccharolyticum* strain HG8, were originally provided by Prof. A.L. Demain of MIT. Both have been cultured for over ten years at the Thayer school. Inocula were grown up in test tubes or small serum vials. Inocula of *C. thermocellum* were grown up using avicel as carbon source, *C. thermosaccharolyticum* were grown up using xylose.

Medium Formulation:

All medium ingredients were reagent grade or better and were supplied by Sigma (St. Louis, MO) or VWR Scientific (Boston MA). The basic medium formulation consisted of: paper sludge and/or xylose at varying concentrations, 1.5 g/L ammonium chloride, 2 g/L citric acid tripotassium salt, 1.25 g/L citric acid free acid monohydrate, 1 g/L sodium sulfate, 1 g/L potassium phosphate monobasic, 2.5 g/L sodium bicarbonate, 1 g/L magnesium chloride hexahydrate, 0.2 g/L calcium chloride dihydrate, 0.1 g/L ferric chloride tetrahydrate, 1 g/L or less L-cysteine hydrochloride monohydrate, 20 mg pyridoxamine dihydrochloride, 4 mg/L

PABA, 2 mg/L d-biotin. For growth of C. *thermosaccarolyitcum*, 5 mg/L thiamin was added. For higher substrate concentration experiments and the continuous culture studies, 5 g/L yeast extract was also added. At high substrate concentrations, 5 g/L urea was added to provide extra nitrogen. Batch experiments also included additional MOPS at up to 10 g/L. Paper sludge was kindly supplied by the James River Corp. (currently Crown Vantage) paper mill in Berlin NH. The paper sludge was stored either frozen or refrigerated until used. For sterilization, paper sludge was autoclaved at 121°C for at least 60 minutes when in small quantities (100mL) and more than 10 hours when in large quantities (18L). The medium nitrogen, buffer and mineral solutions were autoclaved in separate bottles and then combined when cool. Vitamins were filter sterilized through a 0.2 μm filter (Gelman Sciences, Ann rbor MI) and added to the other nutrients.

Growth conditions:

Batch experiments were carried out in anaerobic 250 ml serum vials. Samples were taken periodically by syringe. Pressure inside the bottle was relieved with a sterile needle and filter. Gas production was measured to an approximate degree by capturing vented gasses in a graduated cylinder by water displacement. The vials were allowed to grow in an incubator at 55 or 60°C and were agitated occasionally by manually inverting the vial a few times.

Fed-batch growth was carried out in a 3.0 L, pH controlled glass reactor (Applicon Dependable Instruments, Foster City, CA). The reactor was kept at between 55 and 60 °C and the pH was controlled at 6.75, plus or minus 0.2 using 15% KOH. The reactor was fed periodically with 70 to 80 ml of 200 g/L sterile paper sludge. The paper sludge was sterilized by being being packed into open ended 10 ml syringes and autoclaved for one hour. Usually 7 plugs of sludge, each between 10 and 11 ml, were added to the reactor at one time. The exact quantity of the addition was determined by weighing the syringes before and after discharging the sludge into the reactor. Regular agitation was at a slow rate (< 100 rpm)but after each sludge addition, the reactor was agitated at > 600 rpm to disperse the plugs of paper sludge. The working volume of the reactor was kept in the vicinity of 1 L by periodically drawing 20-50 ml samples.

Continuous growth was carried out in a 1.5 L working volume, pH controlled, stirred tank reactor agitated at 300 rpm. The reactor was kept at 56°C plus or minus 1° and the pH was controlled at 6.75, plus or minus 0.5. A sterile sludge slurry of between 17 and 20 g solids/L was added periodically in 7 ml aliquots. The sludge delivery was controlled with an Allen-Bradley (Milwaukee WI) SLC 500 programmable logic controller and delivered by a pneumatically actuated sterile BEC Isolok SAA157-47-44 sampling piston (Bristol

Equipment Company, Yorkville IL) drawing from a 30 L, sterile, stainless steel storage vessel (see figure 2). To refill the slurry storage vessel during an experiment, an 18L carboy filled with sterile sludge slurry was attached to the storage vessel using a steam sterilized 1 in. ID flexible hose. Agitation of the slurry reservoirs was provided by an overhead drive system (Bellco Glass, Vineland, NJ) and stainless steel impeller. Nutrient additions were made to the reactor by periodically pumping in a small volume of a concentrated nutrient solution. Typically two reactors were used in parallel, drawing off of the same feed storage vessel.

Analytical Methods:

Dry weight of the paper sludge was determined by drying samples in a 70 °C oven for several days. For batch experiments, post reaction dry solids concentration was determined by filtering or centrifuging to remove solids from the broth and then drying at 70 °C. For continuous experiments, dry weights of samples were determined without removing the supernatant.

Ethanol, organic acid and sugar content of the fermentation broth was determined by HPLC. The HPLC apparatus consisted of: an Alcott model 728 auto sampler (Allcott Chromatography, Norcross GA), a pump (Milton Roy, Riviera Beach, FL), a VICI EQ-60 injector valve (Valco Instruments, Houston TX), an Altex T56 RI detector (Beckman Instruments, Palo Alto CA). The column used was a Biorad (Richmond CA) HPX-87H operating at 0.5 mL/minute.

Initial and final cellulose content of the dry solids was determined by quantitative saccharification via acid hydrolysis (2 hr at 30°C, 65 % H_2SO_4 followed by 1 hr at 121°C, 4.5 % H_2SO_4) and either enzymatic glucose assay or HPLC. The enzymatic glucose assay consisted of a coupled reaction catalyzed by 100 units each of glucose-6-phosphate dehydrogenase and hexokinase taking place in a buffer containing 250 mM tris HCl pH 8.0, 1 mM ATP disodium salt, 1.5 mM NAD grade III-C, and 2.1 mM $MgCl_2 \cdot 6H_2O$ (all Sigma) to give an absorbance at 340nm proportional to glucose concentration. Absorbance was measured with a Spectronic 21 spectrophotometer (Milton Roy, Riviera Beach, FL) set to the lowest reading possible plus 0.05. Degradation of samples through hydrolysis was corrected for by running standard samples of avicel, hardwood flour and xylose for each batch of quantitative saccharification experiments.

RESULTS

Batch studies

Batch studies were carried out to get an initial indication of how well a coculture of *C. thermocellum* and *C. saccharolyticum* could convert paper sludge. It was found that the thermophiles grew well on paper sludge using a medium developed for use on other substrates such as avicel. Batch studies were done in the absence of pH control and growth usually halted before full conversion was achieved. Some paper sludges have a strong buffering capacity at neutral pH but others do not, thus the sludge itself could not be relied upon to buffer the reaction.

The effect of paper sludge concentration was investigated. Above 20-40 g/L solids, paper sludge becomes difficult to agitate using conventional laboratory equipment. When concentrated by vacuum filtration to 200 g/L solids, paper sludge takes the form of a damp solid and appears to be unsuitable as a substrate for submerged culture growth. An experiment was run to test the capabilities of batch culture at different sludge concentrations. Figures 3 to 7 show results from batch experiments carried out at sludge concentrations of between 20 and 130 g/L sludge solids, 10 and 65 g/L cellulose, supplemented with xylose.

Figures 3 and 4 show the ethanol and acetate concentrations through the experiment for the different sludge concentrations. The more concentrated vials were difficult to sample, especially at the beginning of the experiment and thus data are scarce for these reactors. Rapid growth and product production was seen at all substrate concentrations and was mostly complete by 150 hours for all vials. There appeared to be no inhibitory effect of high sludge concentrations

Figure 5 shows the ethanol selectivity ratio and conversion versus initial sludge concentration. There is some scatter in the data but there appears to be no trend of change in selectivity with changing concentration of substrate. As the experiment progressed through time, the ethanol selectivity also demonstrated no overall monotonic trend.

The conversion data in figure 5 are based on soluble product concentrations and theoretical yields of CO_2. Measured volumetric gas production (data not shown) was in accord with stoichiometric predictions based on soluble product concentrations. Cell and protein concentrations were not measured and were not accounted for in the conversion calculations. Assuming a cell yield of 10% of utilized substrate, the actual conversion values should be slightly higher than shown in Figure 5. Figure 5 clearly shows that conversion decreases with increasing sludge concentration up to 40 g/L sludge cellulose, after which it appears to level out at about 30% carbohydrate conversion. This may indicate that some beneficial component

of the sludge, perhaps its pH buffering capacity, was depleted when conversion reached 30% or so. This is supported by the observation that the lowest conversion was achieved in the vials with the most xylose: 40 g/L sludge cellulose and 20 g/L xylose.

Fed Batch Study:

pH controlled fed batch studies were done to test the ability of the coculture to grow at higher sludge concentrations without the limitations imposed by pH changes. Results from one experiment are shown in figure 6. It can be seen that the ethanol concentration rose to about 11 g/L and then stopped rising. Acetate production continued at a slow rate after ethanol production had halted. The overall rate of conversion slowed drastically when ethanol production ceased.

Data in figure 7 are drawn from the same experiment as figure 6 and show the accumulated concentration of the sludge cellulose added and the amount of solubilized cellulose accounted for by the sum of the fermentation products and measured soluble sugars. It appears that the culture was successfully hydrolyzing and converting the paper sludge up until it reached about 30 g/L converted, at which point solubilization leveled off and conversion declined in an inverse response to the rising feed concentration. The dip in accumulated sludge concentration at time 130 was caused by the dilution of the reactor with an addition of supplemental nutrients. These nutrients were added in the hope of restoring growth but appear to have had little or no effect toward this end.

Continuous Culture Studies

Continuous experiments were carried out growing a coculture of *C. thermocellum* and *C. thermosaccharolyticum* on 17 to 20 g/L paper sludge (approximately 10g/L cellulose, 2.7 g/L xylan) supplemented with 5 g/L xylose. Steady states were achieved in different experiments at between 20 and 24 hour residence times. Experiments were normally conducted with two reactors arranged in parallel. One experiment was done with the reactors arranged in a cascade series. Steady state operation could be maintained for up to ten days and process upsets, such as such as pH control or feed difficulties, usually resulted in only temporary variance from steady state. Lactic acid production was low and ethanol selectivity was normally between 1.4 and 1.7 (g ethanol /g organic acids). In one experiment ethanol selectivity reached as high as 3.6. Effluent xylose concentrations were low throughout the duration of the experiments and xylose conversion was on the order of 96-98%. Figure 8 shows steady state data for ethanol and acetate production in two parallel experiments. The dip in the performance of one of the reactors was caused by a mal function in pH control . It can be seen that the reactor returned to its initial steady state after the upset.

Dry samples from steady state periods of the experiments were analyzed for residual cellulose and xylan content. The samples used represented the total dry content of the reactor, including the solids and the supernatant. Under steady state operation, cellulose conversion was on the order of 90% and xylan conversion about 70%. Cell mass in the reactors was not measured directly. If a cell yield of 10% on utilized substrate is assumed, as estimated from product concentrations, the carbon balance closes to between 82 and 101%. Much of the variation in carbon balance closure is likely due to the difficulty in estimating actual carbohydrate concentration in the sludge feeding to the reactor. Table I lists feed concentration, soluble product and conversion numbers from different continuously fed experiments.

Variations of the base used for neutralization showed no effect on the steady state. In figure 8 the reactor P2 had it's source of hydroxyl ions switched from KOH to Ca(OH)2 at time 100 for a duration of four residence times. Apart from problems associated with feeding the Ca(OH)2 slurry, there was no apparent effect on the reactor performance.

It some experiments it was observed that once steady state at high conversion was achieved it was no longer necessary to have the feed to the reactor reduced with cysteine.

When cysteine was omitted under high conversion steady state there was no apparent effect on reactor performance.

In one experiment, a cascade reactor was set up in an attempt to more fully convert the substrate. It was found that at a 24 hour residence time in the primary reactor, further processing of the broth in the cascade reactor increased product conversion only slightly and ethanol concentration not at all. At a 12 hour residence time in each reactor (24 hour total) conversion in the primary reactor plummeted and final conversion in the cascade reactor also suffered. See figure 9.

DISCUSSION

This project has demonstrated that paper sludge can be converted directly to ethanol by thermophilic bacteria. The initial rates for the batch experiments appear to be roughly equal regardless of substrate concentration. This indicates that the rate of DMC of paper sludge is not dependent on the solids concentration and that the organisms are unlikely to be inhibited by the higher concentrations of the sludge itself. Batch culture of paper sludge is still difficult at high concentrations, however, since pH control is difficult when mixing and agitation are restricted. This upholds the hypothesis that the sludge is supplying something to the reaction in addition to carbohydrate since the vials with 40 g/L cellulose had a high concentration of xylose (20 g/L) which brought their total carbohydrate concentration equal to 60 g/L -- the same as the 55 g/L cellulose, 5 g/L xylose vials which achieved higher conversion.

Fed batch growth proved to work quite well as a solution to the pH control problem. By adding the sludge gradually, the reactor contents were kept fluid and easy to mix. The maximum ethanol concentrations achieved to date were reached using the fed batch system, which is consistent with findings by other workers[9,14]. The periodic addition of nutrients, particularly nitrogen, has been reported by others as necessary for conversion of high substrate concentrations in fed batch systems[9,14]. While this may be true in some cases, it appears that nutrient limitation was not the problem in this study because nutrient additions to the fed batch experiment represented in figures 8 and 9 failed to restore growth and conversion. The slowing down of growth illustrated in figures 8 and 9 may have been a result of inhibition by the accumulated fermentation products or potassium originating from base additions of KOH, or possibly limitation for a nutrient component not included in the nutrient supplementation.

One of the principal difficulties with using paper sludge as a microbial substrate is the physical handling of the material. The fed batch experiments were manually fed with sterile

sludge. This was cumbersome and posed a considerable risk of contamination of the reactor. The feed devices used for continuous feeding worked well at low sludge concentrations (< 20 g/L), but did not allow for higher concentration feed and thus were of no utility for fed batch or high concentration continuous work.

Work on paper sludge has shown also that the thermophiles are capable of growing in conditions more representative of what they would be likely to encounter in a commercial setting. Batch experiments showed no apparent inhibition in the presence of high concentrations of paper sludge. In fed batch and continuous experiments the thermophiles grew well in the presence of stainless steel reactor components. Continuous operation was able to maintain strong and steady growth over a period of several weeks. Steady states in continuous culture were affected only temporarily, if at all, by a wide variety of process upsets, including changes in feed rate, pH and degree of reductance, the type of base used for neutralization (KOH or $Ca(OH)_2$), moderate pH shocks and short term interruptions. The continuation of this project will investigate the effect, if any, of growing thermophiles in a reactor made entirely of stainless steel. To date there has been no evidence of inhibition due to corrosion products.

One often cited advantage of thermophiles is their ability to simultaneously achieve high conversion of multiple carbohydrate species (cellulose, hemicellulose, soluble sugars). In this project, this advantage has been demonstratewd on a potentially commercial substrate: a continuously fed coculture of *C. thermocellum* and *C. thermosaccharolyticum* simultaneously fermented the sugar polymers contained in paper sludge along with additional monomer xylose in a reasonable time (24 hours) with endogenous production of all required saccharolytic enzymes.

CONCLUSIONS

The paper sludge used in this study was a good substrate for production of ethanol by DMC. Observed growth rates, product concentrations, ethanol selectivity and extent of conversion were similar or superior to reported values on other potentially commercial substrates. Continued work is needed on handling of sludge solids and achieving higher product concentration levels.

ACKNOWLEDGMENTS

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Figure 1

Utilization of Paper Sludges Allows Process Consolidation

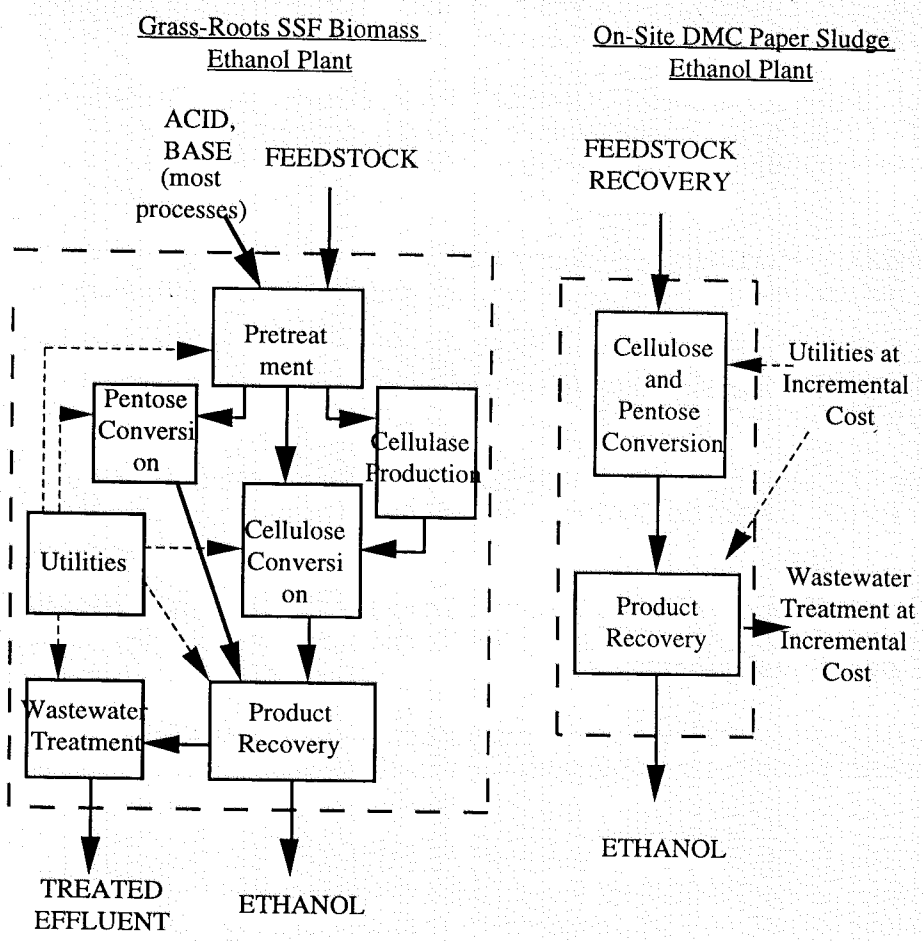


Figure 2

Apparatus for Supplying Continuous Feed of Sterile Paper Sludge to Direct Microbial Conversion Fermentors

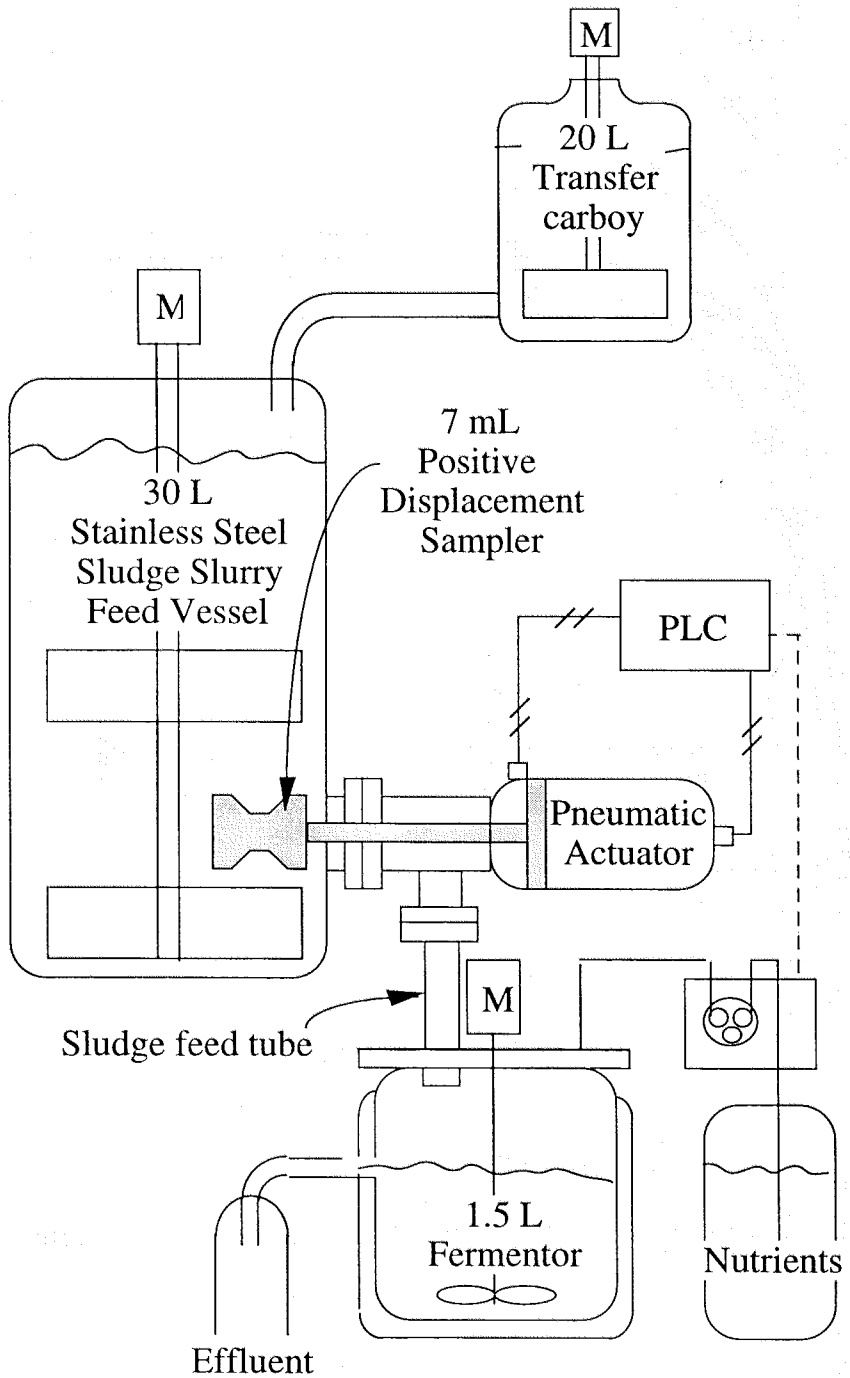
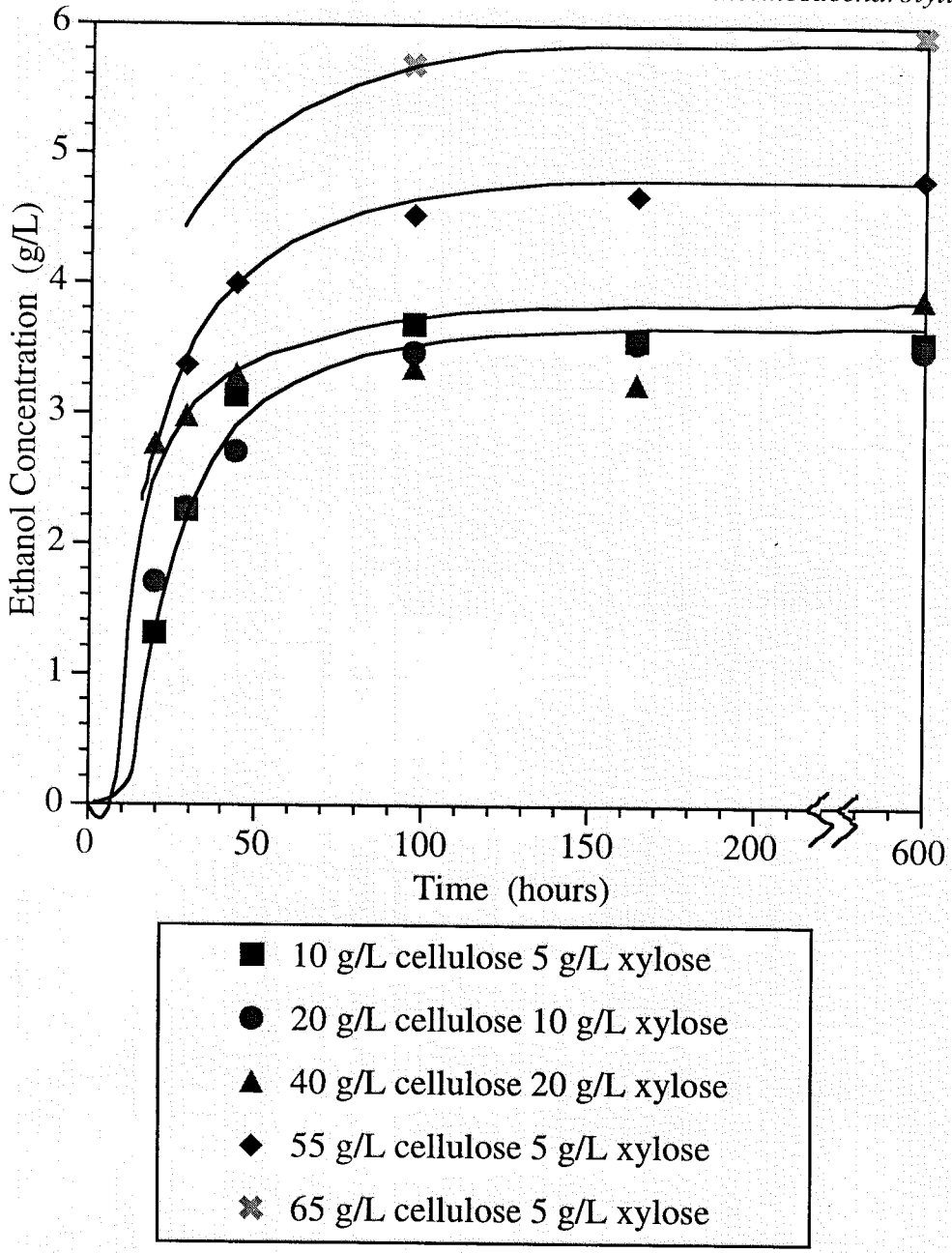


Figure 3

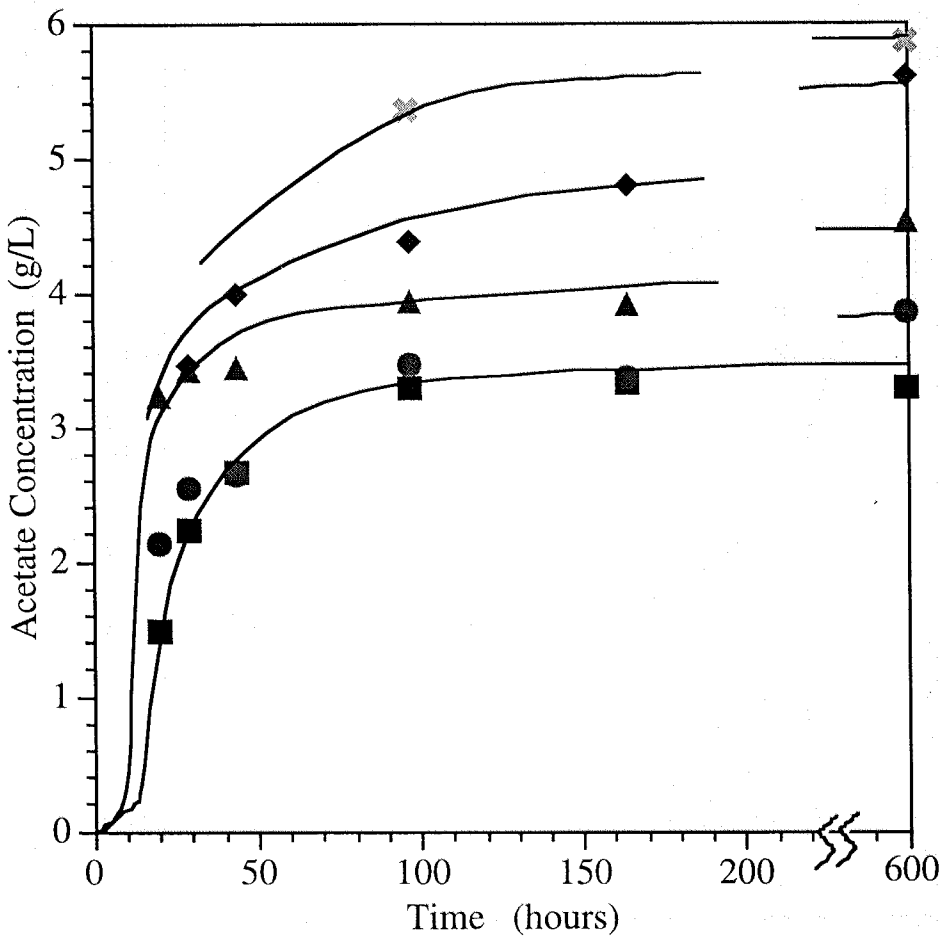
Ethanol concentrations in Batch DMC of Paper Sludge Supplemented with Xylose Using a Coculture of Clostridium *thermocellum* and*Clostridium thermosaccharolyticum*



Symbols represent averages of 2 or 3 replicate experiments.

Figure 4

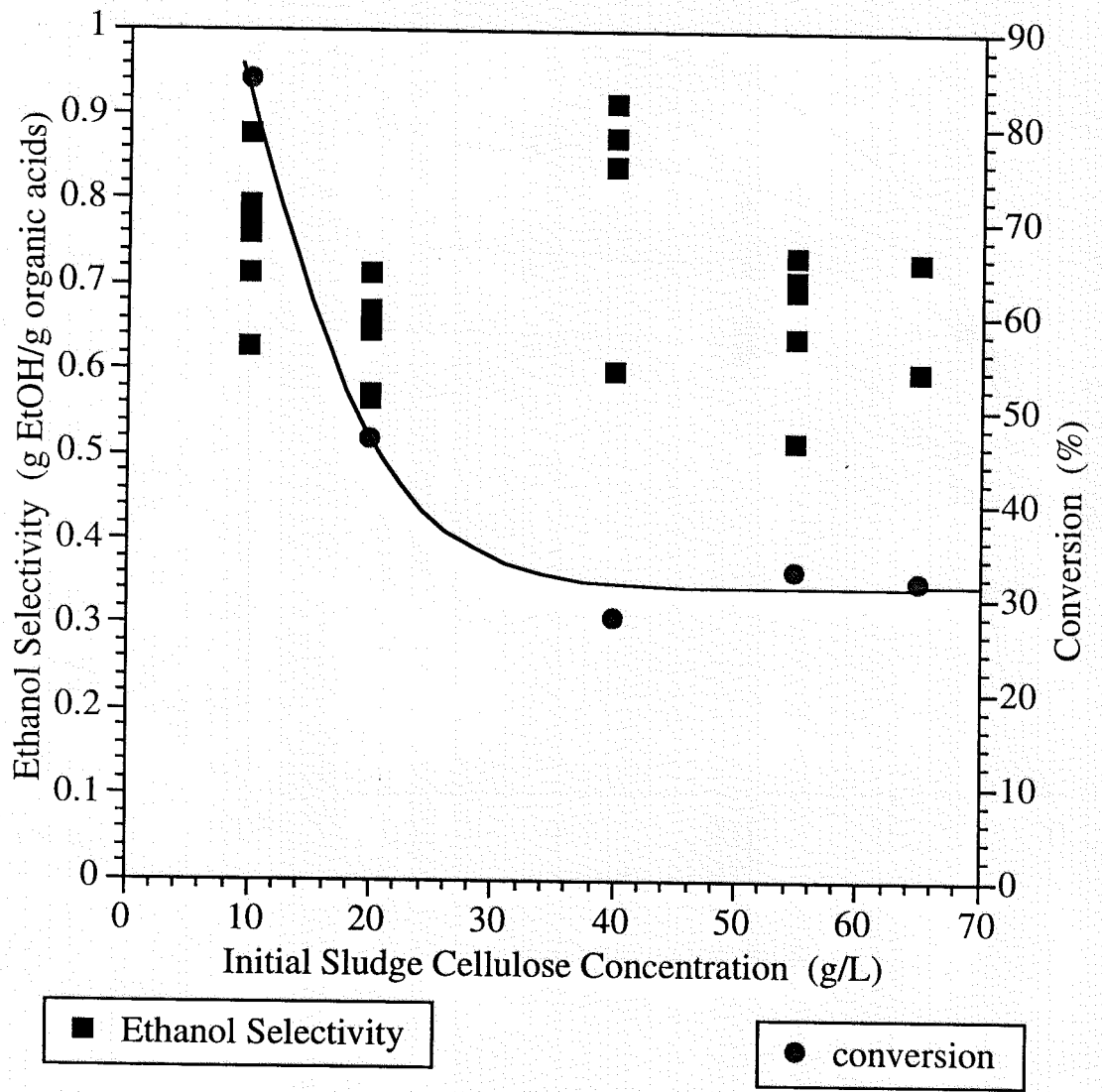
Acetate Concentrations in Batch DMC of Paper Sludge Supplemented with Xylose Using a Coculture of Clostridium *thermocellum* and*Clostridium thermosaccharolyticum*



Symbols as in figure 3

Figure 5

Ethanol Selectivity and Conversion Versus Initial Sludge Concentration for Batch DMC of Paper Sludge Supplemented with Xylose Using a Coculture of *Clostridium thermocellum* and *Clostridium thermosaccharolyticum*



Symbols each represent values calculated from 2 or 3 replicate experiments

Figure 6

Soluble Product Concentration During Fed Batch DMC of Paper Sludge using a Coculture of Clostridium *thermocellum* and*Clostridium thermosaccharolyticum*

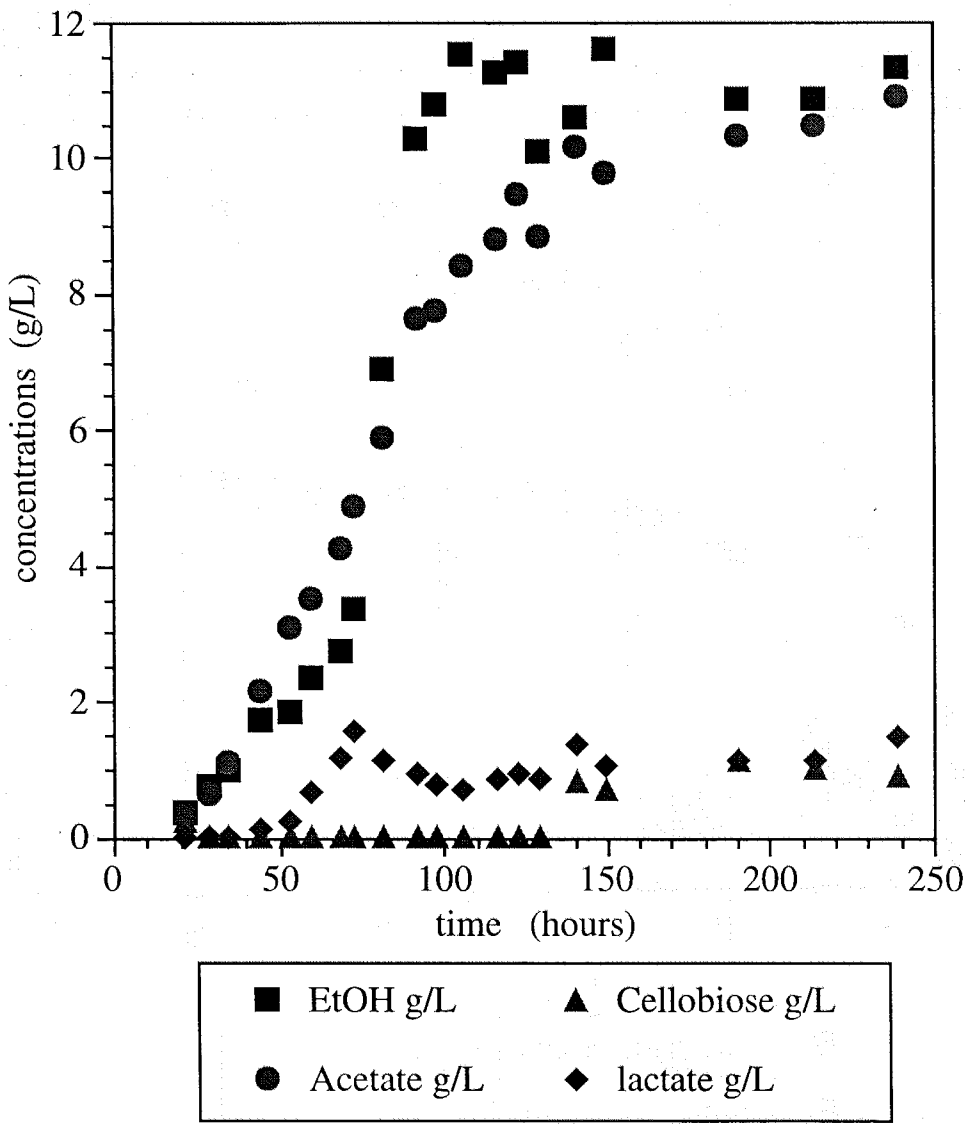


Figure 7

Cumulative Cellulose Substrate Loading and Substrate Conversion During Fed Batch DMC of Paper Sludge Using a Coculture of Clostridium *thermocellum* and*Clostridium thermosaccharolyticum*

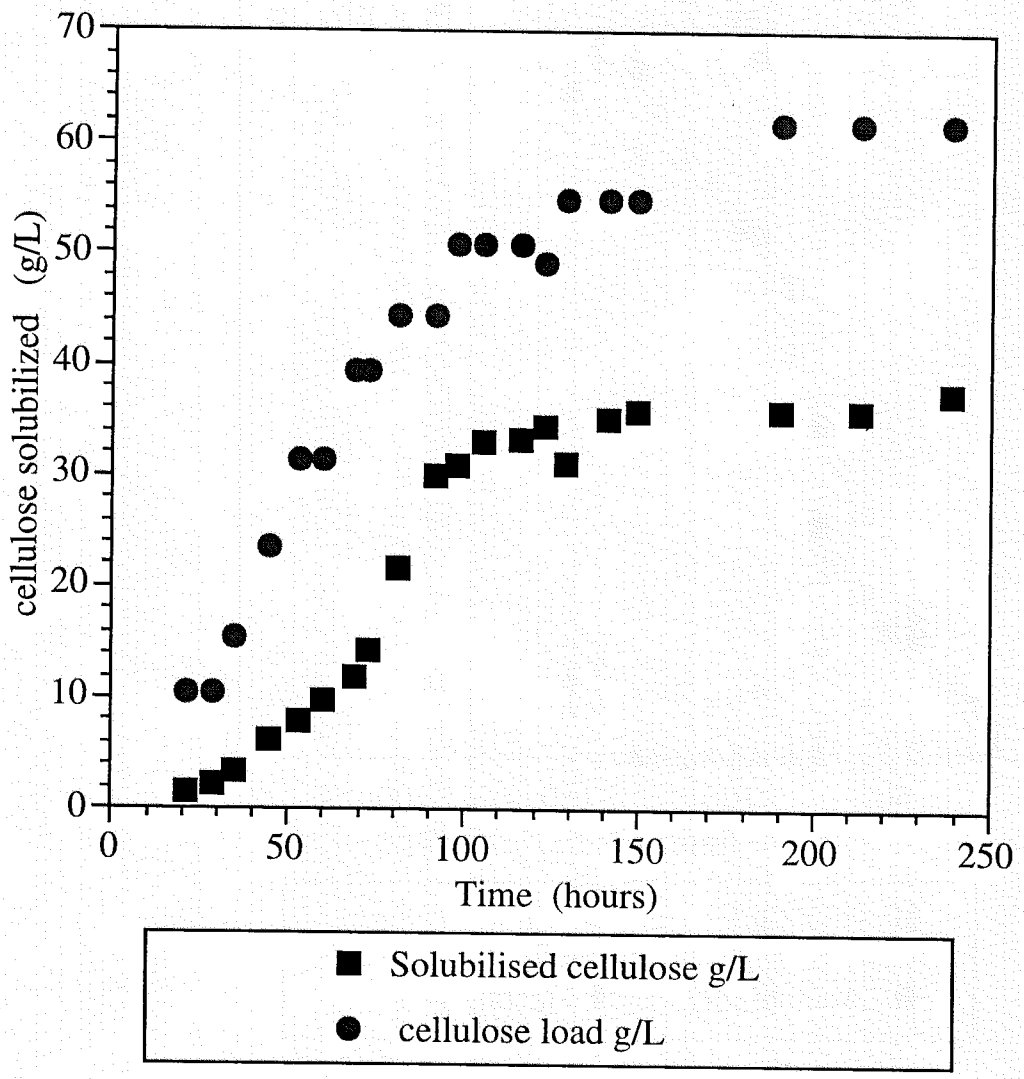
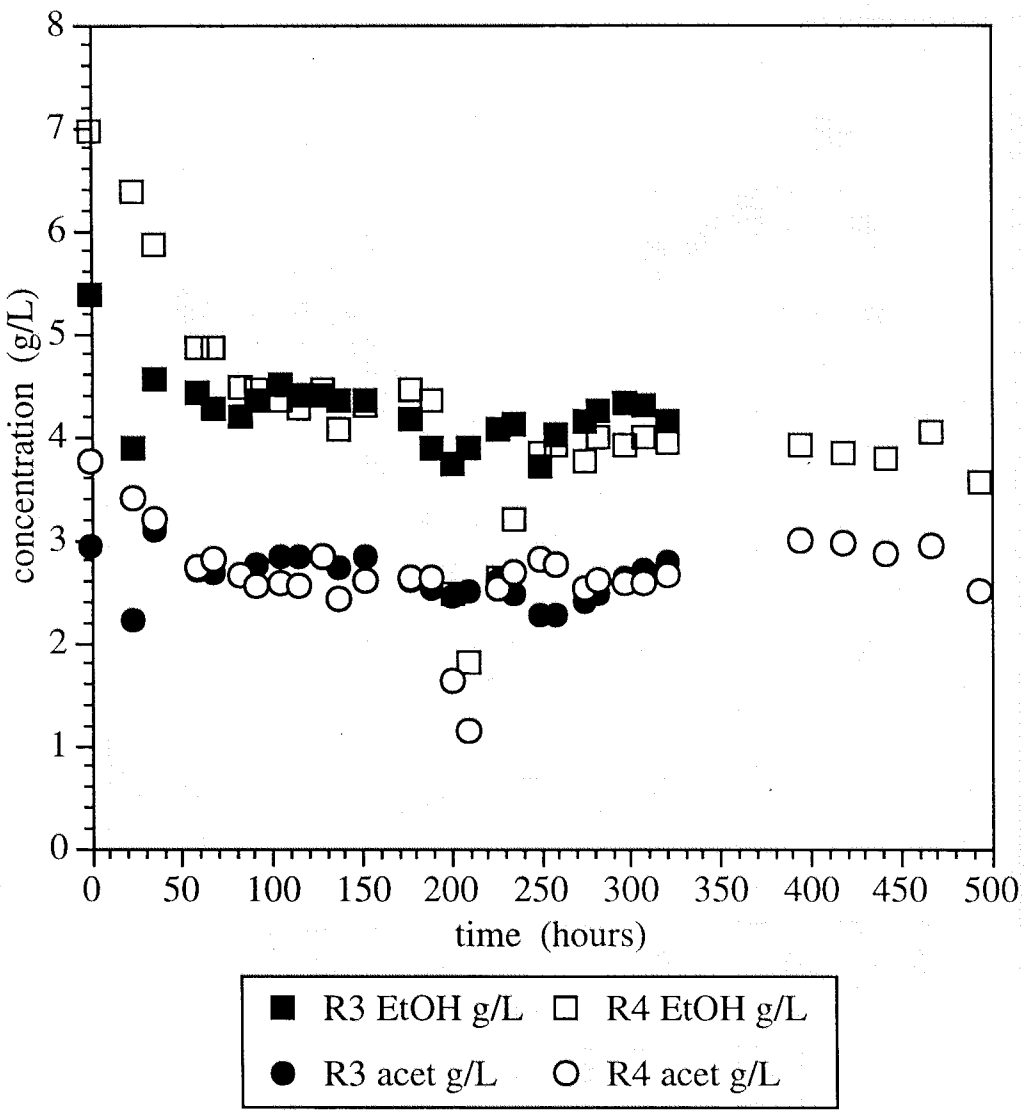


Figure 8

Ethanol and Acetate Concentrations in Continuous DMC of 17 g/L Paper Sludge Supplemented with 5 g/L Xylose at a 22 Hour Residence Time Using a Coculture of Clostridium *thermocellum* and *Clostridium thermosaccharolyticum*

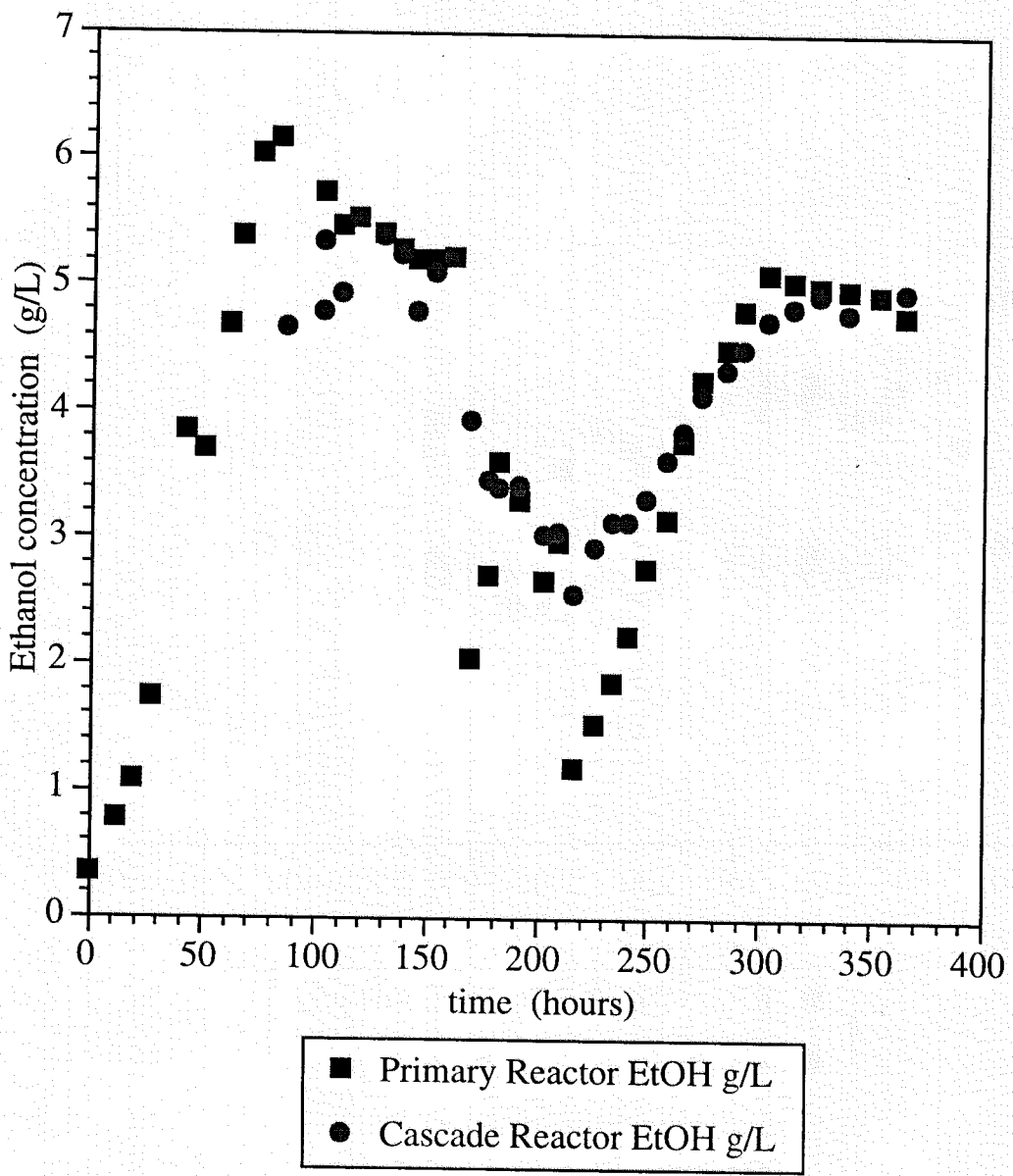


Solid symbols: reactor P1

Open symbols: reactor P2

Figure 9

Ethanol Concentrations in Continuous DMC of 20 g/L Paper Sludge Supplemented with 5 g/L Xylose in a Cascade Reactor System Using a Coculture of Clostridium *thermocellum* and *Clostridium thermosaccharolyticum*



Primary reactor residence time of 24 hours from time 50 to 162 and from time 260 to 353.
Residence time of 12 hours from time 162 to 215.
Batch growth from time 0 to 50 and 215 to 260.

CHAPTER 4
Conclusion and Recommendations

4.1 Conclusions

Work on this project has shown that *C. thermosaccharolyticum* is not inhibited by its own production of ethanol at substrate levels as high as 75g/L. Potassium ion appears to be responsible for the inability of *C. thermosaccharolyticum* to grow at substrate concentrations higher than 75g/L. Calcium hydroxide shows promise as a replacement for potassium hydroxide as a neutralizing agent. Growth of *C. thermosaccharolyticum* in continuous culture has been done reliably and repeatably at 50 and 75 g/L feed xylose over a period of many months. Paper sludge supplemented with xylose has been converted to ethanol and other products in continuous culture with endogenous production of cellulase. Conversion of xylose supplemented paper sludge was on the order of 80 to 90 % in continuous culture. The paper sludge being studied has no apparent inhibitory effect on the growth of *C. thermocellum* and *C. thermosaccharolyticum.*

4.2 Recommendations and Future Work

Because we have still not yet achieved a culture inhibited by its own production of ethanol, work to this end should continue. A variety of approaches are being investigated to circumvent inhibition by potassium and other cations. Work is currently underway to substitute calcium hydroxide, or a combination of different cation bases, for potassium hydroxide. To date continuous culture has been achieved on 50 g/L xylose using calcium hydroxide. Bases with low solubility show extra promise because their ions may never reach inhibitory concentrations in solution. This same low solubility, however, is also a challenge in that it requires more sophisticated delivery systems. Reliability and reproducibility remain unproved with pH control using low solubility base. In the longer term, genetic engineering to eliminate organic acid production is expected to obviate the problem of base ion inhibition.

There is reason to believe that *C. thermocellum* may have ethanol tolerance similar to that of *C. thermosaccharolyticum.* However the ethanol tolerance of *C. thermocellum* has not been investigated with the same rigor as has been done with *C. thermosaccharolyticum.* Since *C. thermocellum* is critical to the DMC process it must be verified that its ethanol tolerance is also suitable for DMC. Of parallel importance is the production of the *C. thermocellum* cellulase in the presence of ethanol and other

fermentation products. These questions relating to *C. thermocellum* will be addressed in the continuation of this project.

Work on thermophilic paper sludge conversion has to this point shown success only at lower concentrations. Demonstration of DMC of paper sludge at higher concentrations remains to be done. The remainder of the project will be devoted toward charaterising performance and establishing reproducibility at low concentrations and utilization of higher sludge concentrations.

4.3 Proposal of Work for NREL Subcontract on Conversion of Paper Sludge to Ethanol.

Task 1: Characterise Continuous Conversion of Paper Sludge to Ethanol

The goal is to produce reproducible and reliable results characterizing the conversion of paper sludge at about 15 g/L carbohydrate to ethanol using a coculture of *C. thermocellum* and *C. thermosaccharolyticum*.. A dilution curve of continuous cultures will be undertaken where conversion, productivity and other qualities of interest are determined at various dilution rates. The effectiveness of intermittent feeding of a continuously operated reactor, potentially approaching sequencing batch operation, will be compared to that of continuous culture.

Task 2: Diagnostic Experiments with *C. thermocellum* grown on Soluble Substrates

To support work on insoluble substrates, soluble substrate work will be conducted using cellobiose as a carbon source aimed at determining: a) strain sufficiency and nutritional feed adequacy at moderate (on the order of 50 g/L) feed concentrations b) ethanol tolerance and c) the effects of ions resulting from neutralization (if necessary).

Task 3 : Continuous Conversion of Paper Sludge at Moderate Concentrations

The goal is to produce reproducible and reliable results characterizing the continuous conversion of paper sludge (at about 50 g/L carbohydrate) to ethanol using a coculture of *C. thermocellum* and *C.*

thermosaccharolyticum. A pH controlled batch experiment will be done in the stainless steel reactor to confirm that the reactor itself is not causing inhibition problems for the culture. Assuming that the reactor material poses no problem, experiments will be carried out on paper sludge using an intermittent feed strategy that will enable good control over substrate feed delivery rates. Results from tasks 1 and 2 will be incorporated into sludge experiments as applicable.

Pending success of the cation investigation and conversion of paper sludge at moderate concentrations, continuous or semi-continuous conversion of still higher concentrations of cellulose will be attempted.

Task 4: Effect of Cation Concentrations Resulting from pH Neutralization on *C. thermosaccharolyticum*

The goal of this task is to surpass the 75 g/L xylose feed barrier and to quantitatively evaluate the tolerance of *C.thermosaccharollyticum* to endogenously produced ethanol. Continuous cultures will be used to establish growth of *C. thermosaccharolyticum* at high xylose concentration using bases other than potassium hydroxide. Calcium hydroxide and mixtures of different bases will be tested.